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The Effect of Protein Kinase C Modulation with Bryostatin 1 on Paclitaxel-Induced Growth Inhibition and Apoptosis in Human Breast Cancer

Combination of Standard Cytotoxic Agents With Polyamine Analogs in the Treatment of Breast Cancer Cell Lines

Introduction

Breast cancer is the most common non-skin cell malignancy in American women and is the second leading cause of cancer deaths in this group (1). Although there are many active cytotoxic chemotherapeutic agents currently available, the efficacy of these agents is limited by tumor cell resistance. Therefore the need for new therapies remains critical. One approach is to examine the addition of novel anti-tumor agents in combination with standard cytotoxic agents. This study utilized the novel agent, Bryostatin 1, in combination with the taxane, paclitaxel. Bryostatin 1 modulates Protein Kinase C (PKC) which is a critical enzyme in cell signal transduction (2). Bryostatin 1 has demonstrated direct anti-tumor activity as well as enhanced the anti-tumor effect of cytotoxic agents (3-6). Paclitaxel has demonstrated significant clinical activity against multiple tumor types including breast (7). The purpose of this study was to evaluate the hypothesis that PKC modulation by bryostatin 1 would augment paclitaxel-induced cytotoxicity in breast cancer cells. These pre-clinical studies were intended to form the foundation for the design of clinical studies in breast cancer patients utilizing combination therapy with paclitaxel and bryostatin 1. When results from this study were negative, a change in direction to focus on combinations of polyamine analogs and chemotherapeutic agents was undertaken.

Body

Experimental Methods

The breast cancer cell lines, MCF 7, T47d, MDA MB 231, MDA MB 468, MDA MB 435 and Hs578t were utilized. These include both estrogen receptor positive and negative cell lines (8). Bryostatin 1 was obtained from the National Cancer Institute and maintained as a 1mM stock in DMSO (stored at -20°C). Paclitaxel was a gift from Bristol-myers/Squibb. A concentrated paclitaxel solution 10mM in DMSO, was stored at -20°C . Docetaxel was a gift from Rhone-Poulenc Rorer, a stock solution was made up in ethanol at 10mg/ml and stored at -20°C . 5-Fluorouracil, vinorelbine, cisplatin were obtained from the oncology pharmacy. 5 Fluorouracil and cisplatin were stored at -20°C and vinorelbine at 5°C . Fluorodeoxyuridine (stock solution 10mM in water, stored at -20°C) and doxorubicin (stock solution 10mM in DMSO, stored at -20°C) were obtained from Sigma Co. Polyamine analogs N^1 -[(cyclopropyl)methyl]- N^{11} -ethyl-4,8-diazaundecane (CPENSpm), and N^1 -[(cycloheptyl)methyl]- N^{11} -ethyl-4,8-diazaundecane (CHENSpm) were obtained from the laboratory of Dr. Robert Casero (Johns Hopkins Oncology Center, Baltimore, MD). CPENSpm and CHENSpm were synthesized by Dr. Patrick Woster (Wayne State University, Detroit MI). The polyamine analogs are dissolved in water for 10mM stock solutions, filter sterilized and stored at -20°C . All

drugs were diluted as required in cell culture medium then added individually to cell cultures using a range of concentrations from 1×10^{-10} M to 1×10^{-6} M (with vehicle treated cultures utilized as controls) to determine the growth inhibition curves for each agent in each cell line. For combination studies, bryostatin 1 was utilized at three different concentrations, 1, 10 and 100 nM.

For growth inhibition studies, exponentially growing cells were plated in triplicate in 24 well or 96 well plates. Cell growth inhibition was determined by assessing % cell number or OD 540 in the treatment group versus control on day 5. Cells were detached with trypsinization and quantitation of cell number was done utilizing a coulter counter. Growth inhibition was also assessed utilizing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye assay. Prior to usage of the MTT assay to obtain experimental data, this assay was directly compared with cell counts and found to be consistent and comparable in measuring growth inhibition by drugs in the breast cancer cell lines. For the MTT assay, the cells were plated in 96 well plates and following completion of the culture period the media was discarded and 100 μ l of MTT (5 mg/ml in culture medium, filter sterilized) was added to each well and the plates were incubated for 4 hours at 37°C. The MTT solution was then removed and the formazan crystals were dissolved in 200 μ l/well of a 1:1 (v/v) solution of DMSO:ethanol and color formation read at OD 540. Results were blanked against wells containing media but no cells for the culture period, and % growth inhibition was calculated by comparison of the treatment groups with the vehicle-treated control cells.

Combination studies were done to determine antagonism, additivity, or synergism. For combination studies where one agent has no growth inhibitory activity when added to the cell cultures, antagonism or synergy can be assessed by any significant change in the concentration of the second agent needed to produce the same level of growth inhibition (as seen with the second agent alone) when the first (inactive alone) agent is added. When both agents being utilized in combination studies were individually cytostatic or cytotoxic, the mathematical model for synergy of Chou and Talalay (9) was utilized. Based on this model, the cell cultures were treated with each drug individually at doses which would inhibit cell growth by 50 % (IC₅₀) and at fixed multiples (2 and 3 times) as well as fractions (0.75, 0.5, and 0.25) of the IC₅₀ dose. The drugs were also combined in these same dose fixed ratios and the results analyzed by the Chou and Talalay method (9). Several different schedules of combined drug exposure were utilized since the timing of drug exposure in combination may influence activity.

Results of the Combination Studies: Bryostatin 1

Initial studies included evaluation of the growth inhibitory effects of bryostatin 1, paclitaxel, and the combination of both in the MCF 7 and MDA MB 468 breast cancer cell lines, *in vitro*. Schedules examined included 30 minute pre-treatment with bryostatin 1 followed by 24 hour treatment with paclitaxel, 24 hour concomitant treatment with both paclitaxel and bryostatin 1, and 24 hour pre-treatment with bryostatin 1 followed by paclitaxel for 24 hours. Bryostatin alone was utilized at concentrations ranging from 10^{-9} M to 3×10^{-7} M. In combination with paclitaxel, bryostatin 1 was tested at three

concentrations, 1, 10, and 100 nM. Paclitaxel was utilized at a concentration range of 10^{-10} M to 3×10^{-8} M. Bryostatin 1 alone at an exposure time of 30 minutes or 24 hours did not result in any significant growth inhibition at any of the tested concentrations. Paclitaxel actively growth inhibited both cell lines with IC_{50} 's in the nanomolar range. Since these concentrations of bryostatin 1 were not growth inhibitory, synergy of the combination could be defined as any significant decrease in the IC_{50} compared with that of paclitaxel alone. These combinations of bryostatin 1 and paclitaxel did not demonstrate any significant change in the IC_{50} and therefore did not demonstrate synergy.

To determine if bryostatin 1 and paclitaxel would demonstrate synergy in other breast cancer cell lines, bryostatin 1 at a concentration of 10 nM was utilized in combination with paclitaxel in MDA MB 435 and Hs578t breast cancer cell lines. The cells were exposed to 24 hours of bryostatin 1 then washed and either DMSO as vehicle control, bryostatin 1, or paclitaxel was added for an additional 24 hours. Again, no synergy was seen when bryostatin 1 was combined with paclitaxel.

To extend the previous studies, additional schedules were evaluated in the MCF 7 and MDA MB 468 cell lines. Bryostatin again at concentrations of 1, 10, and 100 nM were combined with paclitaxel at the concentrations described above. Paclitaxel and bryostatin were evaluated at three different treatment schedules; 1. Concomitant exposure for 120 hours. 2. Bryostatin 1 alone for 24 hours then the addition of paclitaxel and bryostatin 1 for 96 hours. 3. Paclitaxel alone for 24 hours then bryostatin 1 alone for 96 hours. Cell growth inhibition by these treatments was assessed on day 5. Again, no synergy was observed in either cell line under any of the experimental conditions examined.

To further assess whether bryostatin 1 was a promising agent for combination therapy in breast cancer, several additional cytotoxic chemotherapeutic agents were evaluated. These agents included vinorelbine, doxorubicin, cisplatin, and 5-fluorouracil. These agents all have known activity in the treatment of breast cancer and produce growth inhibition *in vitro* of the breast cancer cell lines utilized in these studies. In addition, they differ in their mechanisms of action when compared with each other as well as paclitaxel. For these studies the two breast cancer cell lines, MCF 7 and MDA MB 468 were utilized. Again, three different treatment schedules were utilized, concomitant therapy for 120 hours, bryostatin 1 alone for 24 hours then in combination with drug for 96 hours, and drug alone for 24 hours then bryostatin 1 alone for 96 hours. Bryostatin 1 was added at 1, 10 or 100 nM and the cytotoxic drugs were added at a concentration range, which produced from 0 to greater than 80% growth inhibition. No synergistic combinations were identified with any drug and bryostatin 1 in either cell line.

Background: Polyamine Analogs

Polyamines are essential in both eucaryotic and procaryotic cells for growth and differentiation (10-12). It has been noted that the polyamine pathway is upregulated in tumor tissue (11). The polyamine pathway is therefore a rational target for anti-neoplastic therapy (10). Polyamine analogs are structural analogs of the endogenous polyamines. The polyamine analogs can function similarly to endogenous polyamines in

terms of cell uptake, and regulation of polyamine biosynthesis and metabolism but cannot replace the polyamines' essential role in cell growth and differentiation (13-15). Several polyamine analogs have been evaluated in our laboratory, and have been shown to inhibit the growth of breast cancer cell lines as well as induce programmed cell death (16,17). Inhibition of the polyamine pathway has also been shown to modulate the activity of chemotherapeutic agents (18,19).

Results: Combination Studies with Polyamine Analogs

Evaluation of a different class of agent, i.e. polyamine analogs, in combination with chemotherapy in breast cancer cell lines *in vitro* was evaluated. Initial studies were done in the MCF 7 and MDA MB 468 breast cancer cell lines. The polyamine analogs CPENSpm and CHENSpm were utilized in combination with several chemotherapeutic agents. Treatment schedules evaluated including 120 hour concomitant, polyamine analog alone for 24 hours then analog and drug for 96 hours, and drug alone for 24 hours followed by polyamine analog alone for 96 hours. The chemotherapeutic agents evaluated included doxorubicin, cisplatin, 5-fluorouracil, vinorelbine, paclitaxel, and docetaxel. The polyamine agents alone produce cell growth inhibition and therefore synergy in the combinations was determined by the combination index method by Chou and Talalay .

Results of these studies have been submitted for publication (20) and the manuscript is attached. The results of these experiments are depicted in tables 1 through 4. The first two tables depict the results in the MCF 7 cell lines. Table 1 shows the results with CPENSpm with all six drugs at the three schedules examined. The treatment schedule of drug initially for 24 hours followed by CPENSpm for 96 hours demonstrates synergy at fractional growth inhibitions of greater than 50% for all six drugs evaluated. In contrast, with CHENSpm (table 2) only 5-fluorouracil, vinorelbine and paclitaxel demonstrate synergy. Again the treatment schedule of drug prior to polyamine analog is superior. For the MDA MB 468 cell line (tables 3 and 4), CPENSpm only produces synergy (at fractional growth inhibition of greater than 50%) with vinorelbine. Again, synergy is only observed when the drug precedes the analog. CHENSpm in the MDA MB 468 cell line is shown in table 4. Synergy again is only observed when the drug precedes the analog and only with 5-fluorouracil (for fractional growth inhibition of greater than 50%).

Key Research Accomplishments

- Demonstration of lack of synergy between bryostatin 1 and chemotherapeutic agents in human breast cancer cells.
- Demonstration of synergy between certain cytotoxics and polyamine analogs in human breast cancer cell lines.

Reportable Outcomes:

1. Hahm HA, Dunn VR, Butash KA, Deveraux WL, Woster PM, Casero Jr RA and Davidson NE. Combination of standard cytotoxic agents with polyamine analogs in the treatment of breast cancer cell lines. Submitted 2000.
2. Jackisch C, Hahm HA, Tombal B, McCloskey D, Butash K, Davidson NE and Denmead SR. Delayed micromolar evaluation in intracellular calcium precedes induction of apoptosis in thapsigargin-treated breast cancer cells. Clin Cancer Res 6:2844-50, 2000
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4. Davidson NE, Hahm HA, McCloskey DE, Woster PM and Casero RA Jr. Clinical aspects of cell death in breast cancer: the polyamine pathway as a new target for treatment. Endocr Relat Cancer 6:69-73, 1999.
5. Nass SJ, Hahm HA and Davidson NE. Breast cancer biology blossoms in the clinic. Nat Med 4:761-2, 1998.
6. Hahm HA, Davidson NE, Giguere JK, DiBernardo C and O'Reilly S. Breast cancer metastatic to the choroid. J Clin Oncol 16:2280-2, 1998.

Discussion

Conclusions

The combination of bryostatin 1 and paclitaxel utilizing multiple drug treatment schedules and three different concentrations of bryostatin 1 did not show any synergy in the four different breast cancer cell lines evaluated. In addition, evaluation of four other chemotherapeutic agents (with known activity in breast cancer) in combination with bryostatin 1 did not yield any synergistic combinations. Although I cannot rule out the possibility of synergy of bryostatin 1 and paclitaxel in other breast cancer models or with other drugs, a systematic and rather exhaustive evaluation of combination treatment with bryostatin 1 and paclitaxel (as well as four other chemotherapeutic agents) in several breast cancer cell lines *in vitro* makes it seem unlikely that further study of these in our models would prove fruitful. Also in light of these negative data, no experiments were performed to examine PKC activity/translocation or programmed cell death as originally proposed.

Instead, I concentrated on evaluating the therapeutic potential of another class of novel agents, polyamine analogs, in combination with chemotherapeutic agents in breast cancer cell lines, *in vitro*. In contrast to the studies initially performed with bryostatin 1, several

combinations (with polyamine analogs and chemotherapeutic agents) demonstrate synergy in the MCF 7 and MDA MB 468 cell lines. Scheduling of drug exposures appears critical for synergy with drug preceding analog appearing to be the schedule required to mediate a synergistic response. Additional studies utilizing these combinations in additional cell lines, T47d, MDA MB 231, and Hs578t, are currently underway. These experiments are utilizing both CPENSpm and CHENSpm in combination with the chemotherapeutic agents described above but only one schedule is being evaluated, drug alone for 24 hours followed by polyamine analog alone. Additional studies underway are the evaluation of possible mechanisms underlying the synergy seen when 5 fluorouracil and polyamine analogs are combined. These studies are promising for the potential of polyamine analogs in combination therapy in the treatment of breast cancer and may aid in the rational design of combination therapy of polyamine analogs with chemotherapeutic agents in the treatment of breast cancer.

References

1. Wingo PA, Tong T, and Bolden S. Cancer Statistics, 1995 CA Cancer J. Clin. 145:8-30 1995
2. Nishizuka Y., The family of protein kinase C for signal transduction. JAMA 262:1826-1833, 1989.
3. Gorning, RL, Pearson JW, Beckwith M, and Longo DL Preclinical evaluation of bryostatin as an anticancer agent against several murine tumor cell lines:in vitro versus in vivo activity. Ca Res 52:101-107, 1992.
4. Jarvis WD, Povirk, LF, Turner AJ, Traylor RS, Gewirtz DA, Pettit GR, and Grant S Effects of bryostatin 1 and other pharmacological activators of protein kinase C on 1-[B-D-arabinofuranosyl] cytosine-induced apoptosis in HL-60 human promyelocytic leukemia cells. Biochem Pharm 47: 839-852, 1994.
5. Grant S, Jarvis WD, Swerdlow PS, Turner AJ, Traylor RS, Wallace HJ, Lin P-S, Pettit GR, and Gewirtz D Potentiation of the activity of 1-B-D-arabinofuranosylcytosine by the protein kinase C activator bryostatin 1 in HL-60 cells: association with enhanced fragmentation of mature DNA. Ca Res 52:6270-6278, 1992.
6. Basu A and Lazo JS Sensitization of human cervical carcinoma cells to cis-diamminedichloroplatinum(II) by bryostatin 1. Ca Res 52:3119-3124, 1992.
7. Rowinsky EK and Donehower RC Paclitaxel (Taxol) N Engl J Med 332:1004-1014, 1995.
8. Bates SE, Dickson RB, McManaway ME, and Lippman ME Characterization of estrogen responsive transforming activity in human breast cancer cell lines. Ca Res 46: 1707, 1986.
9. Chou T-C and Talalay P Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Reg 22:27-55, 1987
10. Pegg AE Recent advances in the biochemistry of polyamines in eucaryotes. Biochem J 234:249-262, 1986.
11. Pegg AE Polyamine metabolism and its importance in neoplastic growth as a target for chemotherapy. Cancer Res 48:759-774, 1988.

12. Casero Jr RA and Pegg AE Spermidine/spermine N¹-acetyltransferase-the turning point in polyamine metabolism. *The FASEB Journal* 7:653-661, 1993.
13. Porter CW and Bergeron RJ Enzyme regulation as an approach to interference with polyamine biosynthesis-an alternative to enzyme inhibition *Adv Enzyme Regul* 27:57-82, 1988.
14. Bergeron RJ, Neims Ah McManis JS, Hawthorne TR, Vinson JRT, Bortell R and Ingeno MJ Synthetic polyamine analogues as antineoplastics *J Med Chem* 31:1183-1190, 1988.
15. Edwards ML, Prakash NJ, Stemerick DM, Sunkara SP, Bitonti AJ, Davis GF, and Dumont JA Polyamine analogues with anti-tumor activity *J Med Chem* 33:1369-1375, 1990.
16. Davidson NE, Mank AR, Prestigiacomo LJ, Bergeron RJ, and Casero Jr RA Growth inhibition of hormone-responsive and -resistant human breast cancer cells in culture by n1, n12-bis(ethyl)spermine. *Cancer Res* 53:2071-2075, 1993
17. McCloskey DE, Casero Jr RA, Woster, PM, and Davidson NE Induction of programmed cell death in human breast cancer cells by an unsymmetrically alkylated polyamine analogue. *Cancer Res* 55:3233-3236, 1995.
18. Seidenfeld J, and Komar KA Chemosensitization of cultured human carcinoma cells to 1,3-bis(2-chloroethyl)-1-nitrosourea by difluoromethylornithine-induced polyamine depletion. *Cancer Res* 45:2132-2138, 1985.
19. Basu HS, Pellarin M, Feuerstein BG, and Marton LJ Z-DNA induction by polyamine analogs inversely correlates with their effects on cytotoxicity of cis-diamminedichloroplatinum (II) in human brain tumor cell lines. *Cancer Re.* 16:39-48, 1996.
20. Hahm HA, Dunn VR, Butash KA, Deveraux WL, Woster PM, Casero Jr RA and Davidson NE. Combination of standard cytotoxic agents with polyamine analogs in the treatment of breast cancer cell lines. Submitted 2000.

Combination of Standard Cytotoxic Agents With Polyamine Analogs in the Treatment of Breast Cancer Cell Lines^{1,5}

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E-mail: davidna@jhmi.edu
 - 5 Abbreviations Used are DESpm, N¹, N¹²-diethylspermine (also known as BESpm, N¹, N¹²-bis(ethyl)spermine); DENSPM, N¹-N¹¹-diethylnorspermine (also known as BENSpm, N¹, N¹¹-bis(ethyl)norspermine); CPENSPm, N¹-ethyl-N¹¹-((cyclopropyl)methyl)-4,8-diazaundecane and CHENSPm, N¹-ethyl-N¹¹-((cycloheptyl)methyl)4,8-diazaundecane; BESpd, N¹, N⁸-bis(ethyl)spermidine, c-DDP, *cis*-diaminechloroplatinum (II); 5FU, 5-fluorouracil; FdURd, fluorodeoxyuridine; 4HC, 4-hydroperoxycyclophosphamide; ODC, ornithine decarboxylase; AdoMetDC, S-adenosylmethionine decarboxylase; DFMO, difluoromethylornithine; DMSO, dimethylsulfoxide; MTT, 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide; IC₅₀, fifty percent growth inhibition; CI, Combination Index; PCD, programmed cell death; SSAT, spermidine/spermine N¹-acetyl-transferase; m-AMSA, 4'-(9-acridinylamino)methanesulfon-M anisidide; ER, estrogen receptor α .

ABSTRACT

Polyamines are essential for cell growth and differentiation. Structural polyamine analogs have been shown to have anti-tumor activity in experimental models including breast cancer. The ability of polyamine analogs to alter activity of cytotoxic chemotherapeutic agents in breast cancer models has not been evaluated. This study evaluates the ability of two polyamine analogs, N¹-ethyl-N¹¹-((cyclopropyl)methyl)-4,8-diazaundecane (CPENSpm) and N¹-ethyl-N¹¹-((cycloheptyl)methyl)4,8-diazaundecane (CHENSpm) to synergize with cytotoxics in five human breast cancer cell lines. Antagonism, additivity, or synergy of the combinations was determined using the median effect/combination index model. The chemotherapeutic agents chosen, *cis*-diaminechloroplatinum (II), doxorubicin, 5-fluorouracil, fluorodeoxyuridine, 4-hydroperoxycyclophosphamide, paclitaxel, docetaxel, and vinorelbine, all have anti-tumor activity in breast cancer and represent a spectrum of mechanisms. Three treatment schedules of polyamine analog and cytotoxic were tested in MCF-7 and MDA-MB-468 lines, demonstrating a schedule-dependence of synergistic growth inhibition. Cytotoxic agent alone for 24 hours followed by polyamine analog alone for 96 hours resulted in the most synergistic combinations and the greatest synergy. This schedule was then tested in three additional breast cancer lines, and several synergistic combinations were again identified. Two cytotoxics, vinorelbine and the fluoropyrimidines, showed the most promise in combination with the polyamine analogs. They were able to synergize with one or both polyamine analogs in most of the breast cancer cell lines. CPENSpm was also able to synergize with virtually all cytotoxics in the estrogen receptor α positive MCF-7 and T47D lines. These preclinical data demonstrate a treatment schedule and combinations of polyamine analogs and cytotoxics that will be important to study mechanistically and clinically for breast cancer.

INTRODUCTION

Polyamines are essential for cell growth and differentiation and the finding that polyamine levels are increased in malignant versus normal tissues (1-3) has implicated the polyamine metabolic pathway as a target for anti-neoplastic therapy (2, 4, 5). Investigators have synthesized structural analogs that can mimic the natural polyamines in their self-regulatory role, yet are unable to substitute for polyamines in terms of supporting cell growth and differentiation (5, 6). These analogs have been shown to have anti-tumor activity in multiple experimental model systems including breast cancer (7-14). *In vitro* growth of several breast cancer cell lines is inhibited by several spermine analogs including the n-alkylated symmetrically substituted analogs, N¹, N¹²-diethylspermine (DESPm, also known as N¹, N¹²-bis(ethyl)spermine (BESpm)) and N¹-N¹¹-diethylnorspermine (DENSPM, also known as N¹, N¹¹-bis(ethyl)norspermine (BENSpm)) and the unsymmetrically substituted compounds, CPENSpm and CHENSpm (9, 14). In addition, several of these analogs induce programmed cell death (PCD) in breast cancer cell lines (14).

The polyamine analog, DENSPM, has been evaluated in phase I clinical trials (15, 16). Utilizing a once daily infusion schedule (x 5 days, repeated every 21 days), the drug was well tolerated and gastrointestinal toxicity was the dose limiting toxicity. There was no significant hematologic toxicity, and this schedule is currently being evaluated in phase II studies. Pharmacokinetic analysis using this dosing schedule demonstrated patient plasma concentrations in the micromolar range which is consistent with concentrations required *in vitro* for inhibition of cell growth and induction of PCD. The anti-tumor activity of polyamine analogs in multiple experimental model systems as well as the preliminary clinical data available for the analog DENSPM attest to the therapeutic potential of this class of agents.

Although all of the roles of polyamines in cell proliferation are not known, their capacity to interact with DNA (17) and affect DNA conformation (18) are thought to play a role in their normal cellular function. Therefore, several investigators have evaluated whether depletion of polyamine pools can modulate the activity of DNA-reactive drugs in tumor model systems. These studies have generally combined chemotherapeutic agents with compounds that deplete polyamine pools via inhibition of key biosynthetic enzymes such as ornithine decarboxylase (ODC) or S-adenosylmethionine decarboxylase (AdoMetDC) (19-36). The results of these experiments have been mixed, with some combinations demonstrating synergistic or additive activity and others demonstrating antagonism.

Only two combination studies have been done in breast cancer models. Thomas and Kiang (36) evaluated the activity of anti-estrogens in combination with difluoromethylornithine (DFMO), an inhibitor of ODC in the breast cancer cell line, MCF-7, and demonstrated additive activity of these agents on cell growth inhibition. Das *et al* (35) showed that concomitant, pre- or post-paclitaxel exposure of MCF-7 cells to DFMO resulted in antagonism of paclitaxel-induced cell growth inhibition and apoptosis.

Combined effects of cytotoxic chemotherapeutic agents and polyamine analogs in preclinical models of breast cancer have not been assessed. In this report we evaluate the activity of the polyamine analogs, CPENSpm and CHENSpm, in combination with multiple cytotoxic chemotherapeutic agents currently in use in the treatment of breast cancer in breast cell lines, *in vitro*.

MATERIALS AND METHODS

Compounds, Cell Lines and Culture Conditions.

CPENSpm and CHENSpm were synthesized as described previously (37). For all experiments, a concentrated solution (10mM in water, stored at -20°C) was diluted in medium to desired concentration. 5-FU, c-DDP and vinorelbine were obtained from the Johns Hopkins Oncology Center pharmacy. 5-FU and c-DDP were stored at -20°C and vinorelbine at 4°C . Paclitaxel (a gift from Bristol-Myers/Squibb) was stored at 4°C as a 10 mM solution in dimethylsulfoxide (DMSO). Docetaxel, a gift from Rhone Poulenc/Rorer, was stored at -20°C in absolute ethanol. 4HC was a gift from Dr. O. Michael Colvin (Duke Cancer Center, Durham, NC). 4HC was stored in powder form at -20°C and dissolved in fresh in cell culture medium immediately prior to its use. Fluorodeoxyuridine (FdURd) and doxorubicin were obtained from the Sigma Co. and stored at -20°C in water and DMSO respectively. All drugs were diluted in cell culture medium to desired final concentrations except docetaxel that was initially diluted in water and then cell culture medium. The acquisition and maintenance of the breast cancer cell lines, MCF-7, T47D, MDA-MB-468, Hs578t, and MDA-MB-231 have been previously described (38).

Growth Inhibition Assays.

Cells in the exponential growth phase were plated at 1 to 5×10^4 cells/cm² in 24-well tissue culture plates. After attachment overnight, the medium was changed, and the cells were incubated with or without drugs for the desired exposure times. After 120 hours, the cells were detached by trypsinization and counted using a Coulter Counter.

Percent growth inhibition was determined by comparison of cell number per well in treated versus control cells.

Growth inhibition was also assessed utilizing the 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co.) dye assay (39). For the MTT assay, cells were plated in 96-well dishes and treated as above. Upon completion of the treatment period, the media was discarded and 100 μ l of MTT (5mg/ml in culture medium filter sterilized) was added to each well for 4 hours at 37⁰C. The MTT solution was then removed and the formazan crystals were dissolved in 200 μ l/well of a 1:1 (v/v) solution of DMSO:ethanol for 20 minutes at ambient temperature. Change in optical density was determined at OD540. Results were compared to wells which contained culture medium but no cells, and percent growth inhibition was calculated by comparison of the OD540 reading from treated versus control cells. Drug concentrations that resulted in a fifty percent growth inhibition (IC₅₀) were determined from the plots of percentage growth inhibition versus the logarithm of the drug concentration. All experiments were plated in triplicate wells and were carried out at least twice. Prior to usage of the MTT assay in experiments, its results were validated by direct comparison of results from MTT assay and conventional cell growth assays; results were consistently comparable.

Clonogenic Assay.

For colony formation assay, two hundred MCF-7 cells per 60mm tissue culture dish were allowed to attach overnight. The chemotherapeutic drug of interest was then added on day 0. After 24 hours, media were removed, and the cell monolayer washed with drug- and serum-free media. The cells were then exposed for the remainder of the culture period to media containing the polyamine analog alone. After 10 days, the cell monolayer was washed once with phosphate buffered saline, stained with crystal violet

(0.5% crystal violet in a 3:1 (v/v) mixture of water to methanol), washed with water and allowed to dry at ambient temperature. Visible colonies were counted.

Synergy Studies.

The Median Effect /Combination Index Analysis (CI) (40) was utilized to determine antagonism, additivity, or synergy of combination exposures to both polyamine analogs and cytotoxic drugs. Cell cultures were treated with each agent individually at its IC₅₀ concentration (concentration of drug which resulted in 50% growth inhibition) and at fixed multiples (2 and 3 times) and fractions (0.75, 0.50, and 0.25) of the IC₅₀ concentrations. The agents (polyamine analog and drug) were also combined in these same dose-fixed ratios to determine CI. Antagonism was defined as any CI value above 1, additivity as CI=1, and synergy as less than 1 plus or minus the standard deviation. Experiments were done in triplicate, and each experiment yielded one CI value. Experiments that yielded a CI of less than 1 were repeated at least three times to allow for determination of standard deviation for the CI values obtained. Experiments which yielded CI values of greater than 1 were repeated once if the results were consistent and the CI value shown is a representative value from one of these experiments. CI values are shown only for fractional growth inhibition levels of 0.50 or greater, since dose intensity is known to be important in breast cancer treatment (41).

Treatment Schedules.

Three different treatment schedules were utilized to mimic schedules that are potentially clinically relevant. The first treatment schedule utilized simultaneous exposure to both polyamine analog and cytotoxic drug for 120 hours. In the second treatment schedule, the cells were exposed to 24 hours of cytotoxic drug (starting on day 0). The media was then discarded, the cell monolayer washed once with drug-free media and fresh media containing the polyamine analog was added for the remainder of the

culture period (96 hr). The third treatment schedule evaluated cell exposure to polyamine analog alone for 24 hours followed by removal of the media and addition of media containing both polyamine analog and drug for the remainder of the culture period (96 hr). Sustained exposure to the polyamine analog was utilized in all treatment schedules since other studies have shown that lengthy exposure is necessary for optimal polyamine analog activity (42).

Analysis of Polyamine Content.

The polyamine content of treated and untreated cells was determined by precolumn dansylation, reversed-phase, high-performance liquid chromatographic methods of Kabra *et al* (43).

RESULTS

The estrogen receptor-positive MCF-7 cells and the estrogen receptor-negative MDA-MB-468 cells were chosen for these studies as they are representative of hormone-dependent and -independent breast cancer cells. Six chemotherapeutic agents (c-DDP, doxorubicin, 5-FU, vinorelbine, paclitaxel, and docetaxel) were tested in combination with the two polyamine analogs (CPENSpm and CHENSpm) in both lines using the three different treatment schedules. In addition, FdURd and 4HC were tested using the treatment schedule of cytotoxic drug followed by polyamine analog.

Effects of CPENSpm or CHENSpm and chemotherapeutic drugs on MCF-7 Cells.

The schedule of drug exposure for 24 hours followed by CPENSpm for 96 hours in MCF-7 cells showed a synergistic effect on growth for all eight cytotoxic drugs at a fractional growth inhibition of 0.50 or greater (Table 1). The greatest degree of synergy was seen with the fluoropyrimidines and vinorelbine. In contrast, concurrent exposure to drug and CPENSpm for 120 hours resulted in synergistic growth inhibition only with 5-

FU and vinorelbine at fractional growth inhibition of 0.75. Similarly, the schedule of CPENSpm followed by CPENSpm and drug led to synergistic growth effects only with doxorubicin, c-DDP, paclitaxel, and docetaxel. The degree of synergy and the range of fractional growth inhibitions where synergy was seen were also less with this sequence than those seen with the schedule of drug exposure followed by CPENSpm.

As these studies were performed using the MTT assay, similar studies were performed using the colony formation assay rather than growth inhibition as an endpoint to validate this assay. Figure 1 is a representative graph of the median effect/combo index analysis for FdURd for 24 hours followed by CPENSpm for 96 hours in MCF-7 cells. Synergy was seen with the clonogenic assay and the growth inhibition studies, thereby validating the use of the MTT assay.

Identical studies were undertaken using CHENSpm and cytotoxics in MCF-7 cells (Table 2). Evidence for synergistic interaction was seen only with the sequence of cytotoxic agent followed by CHENSpm. Unlike CPENSpm, synergy was seen with fewer drugs including c-DDP and paclitaxel at a fractional growth inhibition of 0.90, 5-FU and FdURd at a fractional growth inhibition of ≥ 0.75 , and vinorelbine at a fractional growth inhibition of ≥ 0.50 . No synergistic combinations were seen with the treatment schedule of concurrent treatment or CHENSpm followed by the combination of cytotoxic and CHENSpm.

Endogenous Polyamine Levels and Analog Levels in Combination Studies in the MCF-7 Cells.

A key question is whether the observed growth inhibitory effects of combinations of polyamine analogs and cytotoxics simply reflects effects on intracellular polyamines or polyamine analog levels. Therefore, the effect of cytotoxic drugs for 24 hours followed by analog alone for the remainder of the culture period on polyamine levels and

analog levels was assessed. Cells were harvested on day 5 for measurement of polyamines and analogs. In all experiments CHENSpm alone did not substantially perturb endogenous polyamine pools whereas CPENSpm treatment resulted in depletion of spermidine and spermine. Studies using paclitaxel, docetaxel, and 4HC in combination with CHENSpm or CPENSpm showed that these drugs had no effect on polyamine levels alone or in combination with either analog (data not shown). Table 3 shows the results of similar studies with the fluoropyrimidines, doxorubicin, c-DDP, and vinorelbine. None of these drugs altered polyamine levels when used alone. In contrast, 5-FU or fluorodeoxyuridine in combination with CHENSpm or CPENSpm resulted in elevations in both analog levels compared with treatment with CHENSpm or CPENSpm alone. CHENSpm levels were increased 3 to 6 fold, but this increase was not associated with substantial changes in the polyamine pools. CPENSpm levels increased only 1.1 to 3.5 fold, but these changes were consistently associated with further reduction in all three polyamine levels compared with analog alone. For the combination studies with doxorubicin, c-DDP, and vinorelbine, there was no consistent change in intracellular analog levels or polyamine levels for the combination of CHENSpm plus cytotoxic when compared to changes seen with CHENSpm or drug alone. But, like fluoropyrimidines, these agents also demonstrated further depletion of polyamine pools in association with a 1.2 to 2.0 fold increase in CPENSpm intracellular concentrations when combined with CPENSpm. It should be noted that the increase in CPENSpm accumulation is offset by an almost exact reduction in charge-complement of the natural polyamines as previously observed by Bergeron, *et al*, 1988 (44)

Effects of CPENSpm or CHENSpm and chemotherapeutic drugs on MDA-MB-468 cells.

Similar studies were carried out using the estrogen receptor-negative MDA-MB-468 cell line. No evidence for synergy was seen for any treatment schedule or with any chemotherapeutic drug in combination with CPENSpm in this cell line (Table 4). Studies with CHENSpm demonstrated synergy only with treatment with either fluoropyrimidine for 24 hours followed by CHENSpm for 96 hours at a fractional growth inhibition of ≥ 0.75 for 5-FU and ≥ 0.90 for FdURd.

Combination studies using other breast cancer cell lines.

Combination studies using the treatment schedule of cytotoxic drug for 24 hours followed by CPENSpm for 96 hours led to the greatest number of synergistic combinations in both MCF-7 and MDA-MB-468 cells. Therefore this treatment strategy was evaluated in three additional breast cancer cell lines, the estrogen receptor-negative MDA-MB-231 and Hs578t cells and estrogen receptor-positive T47D cells as shown in Table 6. Synergistic growth inhibition was seen with all drugs except doxorubicin in T47D cells and all drugs except for doxorubicin and c-DDP in Hs578t cells. In contrast only one combination demonstrated synergy in the MDA-MB-231 cells, CPENSpm followed by vinorelbine.

Finally, since only the two fluoropyrimidines demonstrated synergy when given before CHENSpm in MCF-7 and MDA-MB-468 cells, the sequence of 5-FU or FdURd for 24 hours followed by CHENSpm for 96 hours was evaluated in MDA-MB-231, Hs578t, and T47D cells. Synergy between 5-FU and CHENSpm was seen in all three lines whereas FdURd and CHENSpm interacted in a synergistic fashion only in MDA-MB-231 and Hs578t cells (Table 7).

DISCUSSION

Polyamine analogs have been shown to have anti-tumor activity as single agents in multiple experimental model systems (7-14). Their ability to modulate response to chemotherapeutic agents is worthy of study. This study addressed the activity of two polyamine analogs, CPENSpm and CHENSpm, in combination with multiple chemotherapeutic agents in breast cancer cell lines. The chemotherapeutic agents used were selected as they 1) have anti-tumor activity in breast cancer, 2) are currently in use in the treatment of breast cancer, and 3) represent a broad spectrum of mechanisms of action. They include alkylating agents (4HC), topoisomerase II inhibitors (doxorubicin), anti-metabolites (5-FU and FdURd), anti-mitotic agents (vinorelbine, paclitaxel, and docetaxel), and the DNA reactive agent, c-DDP, which causes both intra- and interstrand DNA adducts.

Synergistic combinations were identified using one or both of the polyamine analogs in all the cell lines evaluated. There was a schedule dependence for synergy, with the sequence of cytotoxic drug exposure for 24 hours followed by polyamine analog for 96 hours resulting in the greatest number of synergistic combinations as well as the greatest magnitude of synergy for the MCF-7 and MDA-MB-468 cell lines. It is unclear why this schedule is superior even when using diverse chemotherapeutic agents in combination with either polyamine analog in the multiple breast cancer cell lines. Most previous combination studies with DFMO and/or MGBG and chemotherapeutic agents have focussed on treatment with the enzyme inhibitor initially to perturb polyamine pools before drug therapy based on the hypothesis that resultant changes in DNA conformation may allow for greater drug access. Despite its biological rationale, this schedule gave inconsistent results with some studies demonstrating synergism (21, 25, 33) for some DNA directed agents while others demonstrated antagonism (23, 25, 31, 34, 35).

Only two studies published to date have evaluated the activity of combination studies with polyamine analogs and chemotherapeutic agents in *in vitro* tumor model systems. One study (24) combined the spermidine analog N¹,N⁸ bis(ethyl)spermidine (BESpd) with m-AMSA in a human lung cancer cell line to evaluate the induction of topoisomerase II-dependent drug induced cleavable DNA complexes. Unfortunately, it did not address cell growth inhibition or colony-forming ability with combination therapy versus drug alone. Therefore, although there was increased induction of cleavable complexes, subsequent effects on cell growth or plating efficiency were not evaluated. Marverti *et al* (45) studied the effect of the spermine analog, BESpm, and c-DDP on the growth of c-DDP-sensitive and -resistant ovarian carcinoma cells. In the c-DDP-sensitive cell line, concomitant exposure to c-DDP and BESpm demonstrated synergy while the c-DDP-resistant cell line was found to be cross-resistant to BESpm. However when the colony forming ability was evaluated after concurrent treatment with both agents, there was a synergistic interaction as determined by median effect/combination index analysis. Effects of BESpm on polyamine pools, SSAT induction, cell cycle, and mitochondrial membrane potential differed between the two cell lines suggesting that multiple pathways played a role in determining cell sensitivity to this agent. However, only cell cycle effects were evaluated in these two cell lines after treatment with both c-DDP and BESpm, and similar profiles were seen with combination therapy in both. Further experiments are needed to determine mechanisms responsible for the synergy seen with combination therapy in this model system. Of note, c-DDP in our breast cancer models demonstrated synergy in combination with CPENSpm and CHENSpm in the **MCF-7 cell line, and CPENSpm in the T47D cell line.**

CPENSpm and CHENSpm are both spermine analogs with anti-tumor activity and the ability to induce PCD in multiple experimental model systems, yet they

apparently have different mechanisms of action. CPENSpm has been shown to superinduce the catabolic enzyme spermidine/spermine N¹-acetyl-transferase (SSAT) in a number of model systems (7, 10, 46). This superinduction is associated with production of hydrogen peroxide and increased oxidative stress is believed to be an important mediator in the induction of PCD by this agent in select tumor types (47). CHENSpm, however, does not superinduce this enzyme in any model system studied; yet it has significant anti-tumor activity and also induces PCD. In a human lung cancer model, this agent leads to a G2/M cell cycle arrest (47) and alters tubulin polymerization (48). This effect of this drug on tubulin dynamics may play an important role in its anti-tumor activity. These agents demonstrated different spectrums of activities in combination with chemotherapeutic drugs in the breast cancer models.

In the breast cancer cell lines studied, two classes of agents, the fluoropyrimidines and vinorelbine, demonstrated the most activity in combination with CPENSpm or CHENSpm. 5-FU demonstrated synergy in combination with CPENSpm in three out of the five breast cancer cell lines evaluated when drug treatment preceded polyamine analog exposure. 5-FU and/or FdURd demonstrated synergy in combination with CHENSpm in all five cell lines. This class of drugs has not been previously evaluated in combination studies with polyamine analogs although there have been several studies in tumor model systems utilizing DFMO in combination with 5-FU. Kingsnorth *et al* (19) evaluated the activity of 5-FU with or without concomitant DFMO exposure and found there was additive activity of these agents in a human colon cancer cell line. Barranco *et al* (32) evaluated 5-FU activity after 24, 48, or 72 hours of pre-treatment with DFMO in two human gastric cancer cell lines. The results were variable with synergy seen in the 24 and 72 hour pre-treatment groups and antagonism seen after 48 hours pretreatment in the AGS-P parental cell line. The second cell line, AGS-6 (a clonal line derived from the

parental cell line), demonstrated additivity in each treatment group. In another study, Zirvi and Atabek (33) evaluated the activity of DFMO and 5-FU in human colon tumor xenografts grown in primary tissue culture. The cultures were exposed continuously for 7 days to DFMO and treated with 5-FU for 1 hour on day 1 or 2. An additive response was observed. None of these three studies addressed possible mechanisms for the synergistic or antagonistic effects seen with DFMO and 5-FU in combination.

Unlike the studies utilizing 5-FU in combination with DFMO, our results in the breast cancer cell lines with two different spermine analogs demonstrate synergistic combinations with one or both analogs in several of the breast cancer cell lines evaluated. These findings warrant further evaluation in *in vivo* models of breast cancer and examination of the possible mechanisms responsible for synergy. 5-FU is an anti-metabolite with several mechanisms of action including inhibition of thymidylate synthase (TS) and incorporation into DNA and/or RNA (51). One or more of these effects of 5-FU may account for the drug's anti-tumor activity. The effect of polyamine pool depletion resulting from polyamine analog accumulation in the cells on the activity of 5-FU has not previously been evaluated. Evaluation of cell cycle modulation, total intracellular 5-FU content, specific incorporation into DNA and RNA, and TS activity in the presence of the polyamine analog are currently being explored. Since different schedules of treatment or the same schedule in a different cell line can result in different responses (additivity, antagonism, or synergy), evaluation of these changes using different treatment schedules and breast cancer cell lines with variable responses should help identify mechanisms which play a role in a synergistic response.

One of the possible mechanisms, the effect of drug on polyamine analog cellular accumulation and polyamine pool depletion, was extensively evaluated in the MCF-7 cell line. This cell line was chosen since it demonstrated synergy with all cytotoxic drugs

evaluated in combination with CPENSpm as well as several drugs in combination with CHENSpm using the treatment schedule of drug followed by analog. Five out of eight drugs tested led to increased CPENSpm levels in the cells treated with the combination compared with cells treated with analog alone and this increase was associated with further depletion of the polyamine levels. This change may well play an important role in the synergistic response seen with these drugs in combination. Whether the increased level of CPENSpm is due to decreased efflux or alterations in metabolism will need to be evaluated. In addition, it will be interesting to see if this phenomenon occurs as well in *in vivo* model systems as well and how these changes might correlate with effects on tumor growth. A study by Gabrielson, *et al* (52) has shown that SSAT induction in human lung tumor explants occurred in response to treatment with the polyamine analog BENSpm and that this response was tumor specific. It will be important to note if this occurs as well with CPENSpm in *in vivo* combination therapy experiments in breast cancer models and whether further increases in CPENSpm levels are associated with further increases in tumor cell-specific SSAT activity.

Similar studies of intracellular polyamines and CHENSpm levels in MCF-7 cells showed that substantial increases in CHENSpm levels occurred only in combination with the fluoropyrimidines. What role this may play in the synergy that is seen with those agents and CHENSpm remains to be defined. Synergistic responses to drug combinations may be due to more than one mechanism. Thus, the finding of changes in polyamine analog levels in combination studies will need to be evaluated in several other breast cancer cell lines to see if this relationship between synergy and increased intracellular analog levels holds true. Not all synergistic drug combinations in the MCF-7 cell line were associated with changes in polyamine pools or analog levels, suggesting

alternate mechanisms are involved in these synergistic responses with certain chemotherapeutic agents.

The combination of vinorelbine, an anti-mitotic agent that binds tubulin resulting in microtubule depolymerization (53), and CPENSpm demonstrates synergy in all the breast cancer cell lines except MDA-MB-468. But when used with CHENSpm in the MCF-7 and MDA-MB-468 cell lines, vinorelbine was antagonistic in the MDA-MB-468 cell line and synergistic in the MCF-7 cell line. Polyamines may play a role in the natural dynamics of microtubules (49, 50), and some polyamine analogs have been shown induce a G2/M cell cycle arrest and alter tubulin dynamics (47, 48). The positive interaction between polyamine analogs and vinorelbine may be mediated by further modulation of this pathway. It is interesting to note that the taxanes, paclitaxel and docetaxel, which are known to stabilize tubulin polymerization, have been shown to synergize with the polyamine analogs as well, albeit under more limited experimental conditions. Whether these interactions are also mediated via similar pathways is not known.

Finally it is noteworthy that the combination of CPENSpm with virtually all the cytotoxics had synergistic effects in the MCF-7 and T47D cells. Since these drugs represent a spectrum of different mechanisms of action, their extensive ability to synergize with CPENSpm is intriguing. This result implies that the analog may be modifying a common pathway by which all these drugs work to produce an anti-tumor response. It is known that most chemotherapeutic agents induce PCD (54, 55). Polyamine analogs are also known to induce PCD in multiple tumor types including breast cancer (13, 14, 56, 57). Studies utilizing the CPENSpm analog in the H157 human lung cancer cell line have shown that production of oxidative stress via hydrogen peroxide production in the cell due to SSAT induction by this analog is a component of

cell death (47). Whether this pathway is involved in the synergistic response seen with combination therapy in these cell lines remains to be evaluated.

In addition, both the MCF-7 and T47D cell lines are estrogen receptor positive and dependent on estradiol for cell growth. It will be important to determine if other ER positive breast cancer cell lines also demonstrate synergy with CPENSpm and a broad spectrum of cancer chemotherapeutic agents. If this is the case, the role of ER-dependent proliferation pathways in breast cancer cells and their association with the activity of polyamine analogs may be important to investigate. Experiments evaluating the activity of anti-estrogens in combination with polyamine analogs in these breast cancer cell lines would also be warranted.

REFERENCES

1. Porter, C. W., Herrera-Ornelas, L., Pera, P., Petrelli, N. F., and Mittelman, A.
Polyamine biosynthetic activity in normal and neoplastic human colorectal tissues.
Cancer, 60: 1275-1281, 1987.
2. Pegg, A. E. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res.*, 48: 759-774, 1988.
3. Seiler, N., Sarhan, S., Grauffel, C., Jones, R., Knodgen, B., and Moulinoux, J. P.
Endogenous and exogenous polyamines in support of tumor growth. *Cancer Res.*, 50: 5077-5083, 1990.
4. Porter, C. W. and Sufrin, J. Interference with polyamine biosynthesis and/or function by analogs of polyamines or methionine as a potential anticancer chemotherapeutic strategy. *Anticancer Res.*, 6: 525-542, 1986.
5. Bergeron, R. J., Neims, A. J., McManis, J. S., Hawthorne, T. R., Vinson, J. R. T., Bortell, R., and Ingeno, M. J. Synthetic polyamine analogues as antineoplastics. *J. Med. Chem.*, 31: 1183-1190, 1988.
6. Bergeron, R. J., McManis, J. S., Liu, C. Z., Feng, Y., Weimar, W. R., Luchetta, G. R., Wu, Q., Ortiz-Ocasio, J., Vinson, J. R. T., Kramer, D., and Porter, C. W.
Antiproliferative properties of polyamine analogues: a structure-activity study. *J. Med. Chem.*, 37: 3464-3476, 1994.
7. Porter, C. W., Ganis, B., Libby, P. R., and Bergeron, R. J. Correlations between polyamine analogue-induced increases in spermidine/spermine N¹-acetyltransferase activity, polyamine pool depletion, and growth inhibition in human melanoma cell lines. *Cancer Res.*, 51: 3715-3720, 1991.

8. Porter, C. W., McManis, J. S., Casero, Jr. R. A., and Bergeron, R. J. Relative abilities of bis(ethyl) derivatives of putrescine, spermidine, and spermine to regulate polyamine biosynthesis and inhibit L1210 leukemia cell growth. *Cancer Res.*, 47: 2821-2825, 1987.
9. Davidson, N. E., Mank, A. R., Prestigiacomo, L. J., Bergeron, R. J., and Casero, Jr. R. A., Growth inhibition of hormone-responsive and -resistant human breast cancer cells in culture by N¹, N¹²-Bis(ethyl)spermine. *Cancer Res.*, 53: 2071-2075, 1993.
10. Chang, B. K., Bergeron, R. J., Porter, C. W., Vinson, J. R. T., Liang, Y., and Libby, P. R. Regulatory and antiproliferative effects of N-alkylated polyamine analogues in human and hamster pancreatic adenocarcinoma cell lines. *Cancer Chemother. Pharmacol.*, 30: 183-188, 1992.
11. Sharma, A., Graves, D., Porter, C. W., Raghavan, D., and Bernacki, R. J. Antitumor Efficacy of N¹,N¹¹-Diethylnorspermine on a human bladder tumor xenograft in nude athymic mice. *Clin. Cancer Res.*, 3: 1239-1244, 1997.
12. Bernacki, R. J., Oberman, E. J., Seweryniak, K. E., Atwood, A., Bergeron, R. J. and Porter, C. W. Preclinical antitumor efficacy of the polyamine analogue N¹, N¹¹-diethylnorspermine administered by multiple injection or continuous infusion. *Clin. Cancer Res.*, 1: 847-857, 1995.
13. McCloskey, D. M., Yang, J., Woster, P. A., Davidson, N. E., and Casero, Jr. R. A. Polyamine analogue induction of programmed cell death in human lung tumor cells. *Clin. Cancer Res.*, 2: 441-446, 1996.
14. McCloskey, D. M., Casero, Jr. R. A., Woster, P. A. and Davidson, N. E. Induction of programmed cell death in human breast cancer cells by an unsymmetrically alkylated polyamine analog. *Cancer Res.*, 55: 3233-3236, 1995.

15. Creaven, P. J., Perez, R., Pendyala, L., Meropol, N. J., Loewen, G., Levine, E., Berghorn, E., and Raghavan, D. Unusual central nervous system toxicity in a phase I study of N¹N¹¹diethylnorspermine in patients with advanced malignancy. *Invest. New Drugs*, 15: 227-234, 1997.
16. Ettinger, D. S, Bowling, K., Hoker, B., Chen, T. L., Zabelina, Y., Casero, Jr. R. A., Phase I study of N¹,N¹¹ diethylnorspermine (DENSPM) in patients with non-small cell lung cancer (NSCLC) Proc. ASCO abstract, 733, 1998.
17. Balasundaram, D. and Tyagi, A. K. Polyamine-DNA nexus: structural ramifications and biological implications. *Mol. Cell. Biol.*, 100: 129-140, 1991.
18. Snyder, R. D. Polyamine depletion is associated with altered chromatin structure in HeLa cells. *Biochem. J.*, 260: 697-704, 1989.
19. Kingsnorth, A. N., Russell, W. E., McCann, P. P., Diekman, K. A., and Malt, R. A. Effects of α -Difluoromethylornithine and 5-Fluorouracil on the proliferation of a human colon adenocarcinoma cell line. *Cancer Res.*, 43: 4035-4038, 1983.
20. Chang, B. K., Black, Jr. O., and Gutman, R. Inhibition of growth of human or hamster pancreatic cancer cell lines by α -Difluoromethylornithine alone and combined with *cis*-Diaminedichloroplatinum(II). *Cancer Res.*, 44: 5100-5104, 1984.
21. Seidenfeld, J. and Komar, K. A. Chemosensitization of cultured human carcinoma cells to 1,3-Bis(2-chloroethyl)-1-nitrosourea by difluoromethylornithine-induced polyamine depletion. *Cancer Res.*, 45: 2132-2138, 1985.
22. Chang, B. K., Gutman, R., and Black, Jr O. Combined effects of α Difluoromethylornithine and doxorubicin against pancreatic cancer cell lines in culture. *Pancreas*, 1: 49-54, 1986.
23. Seidenfeld, J., Komar, K. A., Naujokas, M. F., and Block, A. L. Reduced cytotoxic

- efficacy for adriamycin in cultured human carcinoma cell depleted of polyamines by difluoromethylornithine treatment. *Cancer Res.*, 46: 1155-1159, 1986.
24. Denstman, S. C., Ervin, S. J., and Casero, Jr. R. A.. Comparison of the effects of treatment with the polyamine analogue N¹,N⁸Bis(ethyl)spermidine (BESpd) or difluoromethylornithine (DFMO) on the topoisomerase II mediated formation of 4'-(9-acridinylamino)methanesulfon-M anisidide (m-AMSA) induced cleavable complex in the human lung carcinoma line NCI H157. *Biochem. and Biophys. Res. Commun.*, 149: 194-202, 1987.
25. Bakic, M., Chan, D., Freireich, E. J., Marton, L. J., and Zwelling, L. A. Effect of polyamine depletion by α -Difluoromethylornithine or (2R,5R)-6-Heptyne 2,5-diamine on drug-induced topoisomerase II-mediated DNA cleavage and cytotoxicity in human and murine leukemia cells. *Cancer Res.*, 47: 6437-6443, 1987.
26. Chang, B. K., Gutman, R., and Chou, T-C. Schedule-dependent interaction of α -Difluoromethylornithine and *cis*-Diaminedichloroplatinum(II) against human and hamster pancreatic cancer cell lines. *Cancer Res.*, 47: 2247-2250, 1987.
27. Shrestha, R. D., Fujimoto, S., and Okui, K. Contradictory antitumor efficacies produced by the combination of DNA attacking drugs and polyamine antimetabolites. *Jpn J. Surg.*, 17: 263-268, 1987.
28. Shaw, M. W., Guinan, P. D., McKiel, C. F., Dubin, A., and Rubenstein, M. Combination therapy using polyamine synthesis inhibitor Alpha-Difluoromethylornithine and adriamycin in treatment of rats carrying the Dunning R3327 MAT-LyLu prostatic adenocarcinoma. *Prostate*, 11: 87-93, 1987.
29. Seidenfeld, J., Barnes, D., Block, A. L., and Erickson, L. C. Comparison of DNA interstrand cross-linking and strand breakage by 1,3-Bis(2-chloroethyl)-1-

- nitrosourea in Ppolyamine-depleted and control human adenocarcinoma cells
Cancer Res., 47: 4538-4543, 1987.
30. Johnson, M., Shaw, M., Rubenstein, M., and Guinan, P. Effect of early and delayed difluoromethylornithine pretreatment upon cyclophosphamide chemotherapy. Clin. Physiol. Biochem., 8: 11-15, 1990.
 31. Hunter, K. J., Deen, D. F., Pellarin, M. and Marton, L. J. Effect of α -Difluoromethylornithine on 1,3-Bis(2-chloroethyl)-1-nitrosourea and cis-Diaminedichloroplatinum(II) cytotoxicity, DNA interstrand cross-linking, and growth in human brain tumor cell lines *in vitro*. Cancer Res., 50: 2769-2772, 1990.
 32. Barranco, S. C., Townsend, Jr. C. M., Ho, B. Y., Reumont, K. J., Koester S. K., and Ford P. J. Schedule dependent potentiation of antitumor drug effects by α -difluoromethylornithine in human gastric carcinoma cell *in vitro*. Invest. New Drugs, 8: S9-S18, 1990.
 33. Zirvi, K. A. and Atabek, U. *In vitro* response of a human colon tumor xenograft and a lung adenocarcinoma cell line to α -Difluoromethylornithine alone and in combination with 5-Fluorouracil and doxorubicin. J. Surg. Oncol., 48: 34-38, 1991.
 34. Desiderio, M. A., Bergamaschi, D., Mascellani, E., DeFeudis, P., Erba, E., and D'Incalci, M. Treatment with inhibitors of polyamine biosynthesis, which selectively lower intracellular spermine, does not affect the activity of alkylating agents but antagonizes the cytotoxicity of DNA topoisomerase II inhibitors. Br. J. Cancer, 75: 1028-1034, 1997.
 35. Das, B., Rao, A. R., and Madhubala, R. Difluoromethylornithine antagonizes taxol

- cytotoxicity in MCF-7 human breast cancer cells. *Oncol. Res.*, 9: 565-572, 1997.
36. Thomas, T. and Kiang, D. T. Additive growth-inhibitory effects of DL- α Difluoromethylornithine and antiestrogens on MCF-7 breast cancer cell line. *Biochem. and Biophys. Res. Commun.*, 148: 1338-1345, 1987.
37. Saab, M. H., West, E. E., Bieszk, N. C., Preuss, C., Mank, A. R., Casero, Jr. R. A., and Woster, P. M. Synthesis and evaluation of unsymmetrically substituted polyamine analogues as modulators of human spermidine /spermine-N¹-acetyltransferase (SSAT) and as potential anti-tumor agents. *J. Med. Chem.*, 36: 2998-3004, 1993.
38. Armstrong, D. K., Gordon, G. B., Hilton, J., Streeper, R. T., Colvin, O. M., and Davidson, N. E. Hepsulfam sensitivity in human breast cancer cell lines: the role of glutathione and glutathione-S-transferase in resistance. *Cancer Res.*, 52: 1416-1421, 1992.
39. Sladowski, D., Steer, S. J., Clothier, R. H., and Balls, M. An improved MTT assay *J. Immunol. Methods.*, 157: 203-207, 1993.
40. Chou, T-C. and Talalay, P. Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme. Regul.* 22: 27-55, 1984.
41. Hryniuk, W., Bush, H. The importance of dose intensity in chemotherapy of metastatic breast cancer. *J. Clin. Oncol.*, 2: 1281-1288, 1984.
42. Bergeron, R. J., Neims, A. H., McManis, J. S., Hawthorne, T. R., Vinson, J. R. T., Bortell, R., and Ingeno, M. J. Synthetic polyamine analogues as antineoplastics. *J. Med. Chem.*, 31: 1183-1190, 1988.

43. Kabra, P. M., Lee, H. K., Lubich, W. P., and Marton, L. J. Solid-phase extraction and determination of dansyl derivatives of unconjugated and acetylated polyamines by reverse phase liquid chromatography: improved separation systems for polyamines in cerebrospinal fluid, urine and tissues. *J. Chromatogr.*, 380: 19-32, 1986.
44. Bergeron, R. J., Hawthorne, T. R., Vinson, J. R. T., Beck, D. E., Jr., and Ingeno, M. J. Role of the methylene backbone in the antiproliferative activity of polyamine analogs of L1210 cells. *Cancer Res.*, 49: 2959-2964, 1989.
45. Marverti, G., Piccinini, G., Ghiaroni, S., Barbieri, D., Quaglino, D., and Moruzzi, M., S. N¹, N¹² Bis(ethyl)spermine effect on growth of *cis*-Diaminedichloroplatinum(II)-sensitive and-resistant human ovarian-carcinoma cell lines. *Int. J. Cancer*, 78: 33-40, 1998.
46. Casero, Jr. R. A., Celano, P., Ervin, S. J., Wiest, L., and Pegg, A. E. High specific induction of spermidine/spermine N¹-scetyltransferase in a human large cell lung carcinoma. *Biochem. J.*, 270: 615-620, 1990.
47. Ha, H. C., Woster, P. A., Yager, J. D., and Casero, Jr. R. A. The role of polyamine catabolism in polyamine analogue-induced programmed cell death. *Proc. Natl. Acad. Sci.*, 94: 11557-11562, 1997.
48. Webb, H. K., Wu, Z., Sirisoma, N., Ha, H. C., Casero, Jr. R. A., and Woster, P. W. 1-(N-Alkylamino)-11 (N-ethylamine)-4,8-diazaundecanes: Simple synthetic polyamine analogues that differentially alter tubulin polymerization. *J. Med. Chem.*, 42: 1415-21, 1999.
49. Phojanpelto, P., Virtanen, I., and Holtta, E. Polyamine starvation causes disappearance of actin filaments and microtubules in polyamine-auxotrophic CHO

- cells. *Nature* 293: 475-477, 1981.
50. Anderson, P. J., Bardocz, S., Camps, R., and Brown, D. L. The effect of polyamines on tubulin assembly. *Biochem. and Biophys. Res. Commun.*, 132: 147-154, 1985.
 51. Valeriote, F. and Santelli, G. 5-Fluorouracil (Fura) *Pharmac. Ther.*, 24: 107-132, 1984.
 52. Gabrielson, E. W., Pegg, A. E. and Casero, Jr. R. A. The induction of spermidine/spermine N¹-acetyltransferase (SSAT) is a common event in the response of human primary non-small cell lung carcinomas to exposure to the new antitumor polyamine analogue N¹,N¹¹-Bis(ethyl)norspermine. *Clin. Cancer Res.*, 5: 1638-1641, 1999.
 53. Budman, D. R. Vinorelbine (Navelbine): A third-generation vinca alkaloid. *Cancer Invest.*, 15: 475-490, 1997.
 54. Lowe, S. W., Ruley, H. E., Jacks, T. & Housman, D. E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell*, 74: 957-967, 1993.
 55. Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E. & Jacks, T. p53 status and the efficacy of cancer therapy *in vivo*. *Science*, 266: 807-810, 1994.
 56. Kramer, D. L., Fogel-Petrovic, M., Diegelman, P., Cooley, J. M., Bernacki R. J., McManis J. S., Bergeron R. J., and Porter C. W. Effects of novel spermine analogues on cell cycle progression and apoptosis in MALME-3M human melanoma cells. *Cancer Res.*, 57: 5521-5527, 1997.
 57. Ha, H. C., Woster, P. A., and Casero, Jr., R. A. Unsymmetrically substituted polyamine analogue induces caspase-independent programmed cell death in Bcl-2-overexpressing cells. *Cancer Res.*, 58: 2711-2714, 1998.

Table 1 Effects of CPENSpm and chemotherapeutic drugs on MCF-7 cells ^a

Schedule:

Concomitant

CPENSpm & drug Fractional growth inhibition ^b

Drug	0.50	0.75	0.90
Doxorubicin	1.13 +/- 0.16	1.04 +/- 0.18	0.99 +/- 0.27
c-DDP	1.08 +/- 0.13	0.95 +/- 0.18	0.88 +/- 0.22
5-FU	0.69 +/- 0.42	0.75 +/- 0.23	1.27 +/- 0.62
Vinorelbine	0.73 +/- 0.37	0.67 +/- 0.29	0.68 +/- 0.27
Paclitaxel	1.16 +/- 0.11	1.12 +/- 0.14	1.12 +/- 0.16
Docetaxel	0.93 +/- 0.07	0.83 +/- 0.25	0.80 +/- 0.38

Drug then

CPENSpm

Drug	0.50	0.75	0.90
Doxorubicin	0.84 +/- 0.08	0.65 +/- 0.09	0.51 +/- 0.08
c-DDP	0.85 +/- 0.11	0.69 +/- 0.13	0.63 +/- 0.19
5-FU	0.58 +/- 0.13	0.49 +/- 0.12	0.42 +/- 0.12
FdUrd	0.67 +/- 0.16	0.54 +/- 0.08	0.47 +/- 0.11
4HC	0.93 +/- 0.12	0.77 +/- 0.16	0.67 +/- 0.22
Vinorelbine	0.30 +/- 0.29	0.32 +/- 0.20	0.43 +/- 0.06
Paclitaxel	0.74 +/- 0.14	0.68 +/- 0.11	0.67 +/- 0.05
Docetaxel	0.82 +/- 0.15	0.66 +/- 0.19	0.54 +/- 0.21

CPENSpm

then both

Drug	0.50	0.75	0.90
Doxorubicin	1.08 +/- 0.27	0.88 +/- 0.18	0.76 +/- 0.13
c-DDP	1.17 +/- 0.19	0.84 +/- 0.07	0.64 +/- 0.07
5-FU	0.88 +/- 0.14	0.81 +/- 0.19	0.85 +/- 0.42
Vinorelbine	0.74 +/- 0.33	0.77 +/- 0.27	0.89 +/- 0.37
Paclitaxel	0.99 +/- 0.13	0.82 +/- 0.14	0.69 +/- 0.15
Docetaxel	0.69 +/- 0.29	0.58 +/- 0.20	0.50 +/- 0.15

^a Results are shown for combination studies using the polyamine analog, CPENSpm and drug at three different treatment schedules.

^b Combination Index (CI) values for fractional growth inhibitions of 0.50, 0.75, and 0.90 in the MCF-7 cell line. Antagonism CI > 1.00, additivity CI = 1.00, synergy CI < 1.00 +/- standard deviation.

Table 2 Effects of CHENSpm and chemotherapeutic drugs on MCF-7 cells^a

Schedule:

Concomitant

CHENSpm & drug	Fractional growth inhibition ^b		
Drug	0.50	0.75	0.90
Doxorubicin	1.18 +/- 0.15	1.08 +/- 0.22	1.05 +/- 0.25
c-DDP	1.37 +/- 0.61	1.14 +/- 0.56	1.10 +/- 0.44
5-FU	1.28 +/- 0.13	1.11 +/- 0.02	1.00 +/- 0.08
Vinorelbine	1.11 +/- 0.22	1.02 +/- 0.14	0.96 +/- 0.11
Paclitaxel	1.22 +/- 0.09	1.27 +/- 0.06	1.34 +/- 0.02
Docetaxel	1.19 +/- 0.14	1.21 +/- 0.10	1.27 +/- 0.04

Drug then
CHENSpm

Drug	0.50	0.75	0.90
Doxorubicin	1.17	1.28	1.43
c-DDP	1.33 +/- 0.38	1.38 +/- 0.36	0.87 +/- 0.11
5-FU	1.06 +/- 0.06	0.87 +/- 0.03	0.73 +/- 0.02
FdUrd	0.92 +/- 0.27	0.55 +/- 0.13	0.45 +/- 0.06
4HC	1.11 +/- 0.18	0.91 +/- 0.18	0.78 +/- 0.22
Vinorelbine	0.77 +/- 0.15	0.64 +/- 0.10	0.54 +/- 0.07
Paclitaxel	1.15 +/- 0.20	0.95 +/- 0.15	0.81 +/- 0.15
Docetaxel	1.19	1.31	1.44

CHENSpm
then both

Drug	0.50	0.75	0.90
Doxorubicin	1.36 +/- 0.12	1.22 +/- 0.06	1.22 +/- 0.06
c-DDP	1.30	1.13	1.06
5-FU	1.44	1.19	1.04
Vinorelbine	1.43 +/- 0.08	1.29 +/- 0.05	1.20 +/- 0.12
Paclitaxel	1.39 +/- 0.11	1.36 +/- 0.11	1.36 +/- 0.18
Docetaxel	1.54	1.55	1.64

^a Results are shown for combination studies using the polyamine analog CHENSpm and drug with the MCF-7 cell line at three different treatment schedules.

^b Combination Index (CI) values for fractional growth inhibitions of 0.50, 0.75, and 0.90 in the MCF-7 cell line. Antagonism CI > 1.00, additivity CI = 1.00, synergy CI < 1.00 +/- standard deviation.

Table 3 Polyamine and polyamine analog levels on day 5 in the MCF-7 cell line^{a, b}

Treatment Groups	putrescine nmol/mg protein	spermidine	spermine	CHENSpm	CPENSpm
control	2.75	26.18	17.05	0	0
5-fluorouracil (5-FU)	2.21	29.15	24.96	0	0
CHENSpm	1.96	28.57	23.82	5.66	0
5-FU then CHENSpm	1.41	16.70	21.66	21.21	0
CPENSpm	11.96	18.45	8.50	0	23.95
5-FU then CPENSpm	0	2.99	8.29	0	35.05
control	2.19	22.49	15.64	0	0
fluorodeoxyuridine (FdUrd)	1.94	16.29	17.87	0	0
CHENSpm	2.09	20.96	19.08	6.55	0
FdUrd then CHENSpm	2.43	11.43	18.82	21.28	0
CPENSpm	9.22	13.32	7.48	0	39.41
FdUrd then CPENSpm	0	1.59	4.29	0	46.10
control	2.90	25.53	15.23	0	0
doxorubicin	2.96	21.62	11.54	0	0
CHENSpm	2.79	26.31	14.81	1.52	0
doxorubicin then CHENSpm	3.07	20.05	11.43	1.15	0
CPENSpm	8.11	12.31	5.64	0	19.54
doxorubicin then CPENSpm	2.24	3.86	1.83	0	25.58
control	2.75	26.18	17.05	0	0
cis-diaminechloroplatinum (II) (c-DDP)	0	20.35	22.40	0	0
CHENSpm	1.96	28.57	23.82	5.66	0
c-DDP then CHENSpm	1.19	19.60	19.92	7.38	0
CPENSpm	11.96	18.45	8.50	0	23.92
c-DDP then CPENSpm	1.31	4.62	4.85	0	32.43
control	6.46	28.36	15.16	0	0
vinorelbine	4.55	20.34	15.12	0	0
CHENSpm	3.17	28.86	22.05	3.51	0
vinorelbine then CHENSpm	4.77	15.59	17.80	6.09	0
CPENSpm	12.61	15.90	7.45	0	17.87
vinorelbine then CPENSpm	3.69	1.68	5.17	0	26.00

^a Cells were treated with drug alone on day 0 for 24 hours then the media was removed and the cell monolayer washed with drug- and serum-free media, then media with or without analog was then added for the remainder of the culture period (120 hours).

^b Polyamine and analog levels are the mean of duplicate cultures from one representative experiment. All experiments were done at least twice and gave similar results.

Table 4 Effects of CPENSpm and chemotherapeutic drugs on MDA-MB-468 cells^a

Schedule:

Concomitant

CPENSpm & drug	Fractional growth inhibition ^b		
Drug	0.50	0.75	0.90
Doxorubicin	1.28 +/- 0.14	1.45 +/- 0.19	1.73 +/- 0.28
c-DDP	0.96 +/- 0.46	1.33 +/- 0.60	1.75 +/- 1.28
5-FU	1.32 +/- 0.44	1.81 +/- 0.60	2.54 +/- 0.97
Vinorelbine	1.15 +/- 0.02	1.10 +/- 0.09	1.13 +/- 0.16
Paclitaxel	1.29 +/- 0.22	1.37 +/- 0.25	1.55 +/- 0.33
Docetaxel	1.17	1.39	1.72

Drug then

CPENSpm

Drug	0.50	0.75	0.90
Doxorubicin	1.33	1.27	1.25
c-DDP	1.22	1.21	1.22
5-FU	0.98	1.19	1.45
4HC	0.90 +/- 0.16	1.00 +/- 0.32	1.15 +/- 0.54
Vinorelbine	0.93 +/- 0.23	0.83 +/- 0.18	0.78 +/- 0.25
Paclitaxel	0.86 +/- 0.32	0.97 +/- 0.07	1.36 +/- 0.48
Docetaxel	1.18	1.14	1.14

Polyamine analog

then both

Drug	0.50	0.75	0.90
Doxorubicin	1.06	1.43	1.95
c-DDP	1.35 +/- 0.14	1.82 +/- 0.10	2.56 +/- 0.30
5-FU	1.53	1.64	1.79
Vinorelbine	0.98 +/- 0.15	1.05 +/- 0.10	1.20 +/- 0.10
Paclitaxel	1.17 +/- 0.28	1.52 +/- 0.45	2.71 +/- 2.15
Docetaxel	1.17 +/- 0.26	1.38 +/- 0.31	2.17 +/- 1.24

^a Results are shown for combination studies using the polyamine analog CPENSpm and drug with the MDA-MB-468 cell line at three different treatment schedules.

^b Combination Index (CI) values for fractional growth inhibitions of 0.50, 0.75, and 0.90 in the MCF-7 cell line. Antagonism CI > 1.00, additivity CI = 1.00, synergy CI < 1.00 +/- standard deviation.

Table 5 Effects of CHENSpm and chemotherapeutic drugs on MDA-MB-468 cells^a

Schedule:

Concomitant

CHENSpm & drug	Fractional growth inhibition ^b		
Drug	0.50	0.75	0.90
Doxorubicin	1.41	1.45	1.51
c-DDP	1.01	1.17	1.36
5-FU	1.40 +/- 0.33	1.28 +/- 0.39	1.21 +/- 0.44
Vinorelbine	0.97 +/- 0.32	0.97 +/- 0.21	0.99 +/- 0.09
Paclitaxel	1.41 +/- 0.14	1.35 +/- 0.13	1.30 +/- 0.11
Docetaxel	1.62	1.50	1.39

Drug then

CHENSpm

Drug	0.50	0.75	0.90
Doxorubicin	1.50	1.33	1.18
c-DDP	1.13	1.11	1.15
5-FU	1.12 +/- 0.19	0.92 +/- 0.01	0.78 +/- 0.12
FdURd	0.94 +/- 0.37	0.85 +/- 0.25	0.85 +/- 0.13
4HC	1.11 +/- 0.04	0.97 +/- 0.04	0.94 +/- 0.12
Vinorelbine	1.16 +/- 0.07	1.03 +/- 0.08	0.94 +/- 0.12
Paclitaxel	2.13	1.87	1.64
Docetaxel	1.14	1.20	1.26

CHENSpm

then both

Drug	0.50	0.75	0.90
Doxorubicin	1.12 +/- 0.19	1.05 +/- 0.13	1.03 +/- 0.11
c-DDP	1.36	1.30	1.24
5-FU	1.32	1.17	1.18
Vinorelbine	1.14 +/- 0.08	1.06 +/- 0.10	0.99 +/- 0.12
Paclitaxel	1.30	1.28	1.26
Docetaxel	0.99 +/- 0.23	0.98 +/- 0.13	0.98 +/- 0.06

^a Results are shown for combination studies using the polyamine analog, CHENSpm and drug with the MDA-MB-468 cell line at three different treatment schedules.

^b Combination Index (CI) values for fractional growth inhibitions of 0.50, 0.75, and 0.90 in the MDA-MB-468 cell line. Antagonism CI > 1.00, additivity CI = 1.00, synergy CI < 1.00 +/- standard deviation.

Table 6 Effects of CPENSp^m and chemotherapeutic drugs on MDA-MB-231, Hs578t, and T47D cells^a

MDA-MB-231 cells		Fractional growth inhibition ^b	
Drug	0.50	0.75	0.90
Doxorubicin	1.21	1.16	1.25
c-DDP	1.07	1.41	1.86
5-FU	1.04 +/- 0.62	0.91 +/- 0.30	1.11 +/- 0.78
FdURd	1.05 +/- 0.32	1.11 +/- 0.27	1.58 +/- 0.51
4HC	1.20 +/- 0.31	1.37 +/- 0.36	0.58 +/- 0.44
Vinorelbine	1.09 +/- 0.06	0.89 +/- 0.07	0.76 +/- 0.10
Paclitaxel	1.16 +/- 0.09	1.08 +/- 0.07	1.04 +/- 0.16
Docetaxel	0.92 +/- 0.22	0.86 +/- 0.25	0.85 +/- 0.31

Hs578t cells		Fractional growth inhibition ^b	
Drug	0.50	0.75	0.90
Doxorubicin	1.06	1.15	1.38
c-DDP	1.17	1.24	1.31
5-FU	0.82 +/- 0.09	0.69 +/- 0.05	0.59 +/- 0.04
FdURd	0.80 +/- 0.29	0.54 +/- 0.15	0.45 +/- 0.07
4HC	1.01 +/- 0.09	0.76 +/- 0.16	0.64 +/- 0.16
Vinorelbine	1.18 +/- 0.27	0.73 +/- 0.16	0.48 +/- 0.06
Paclitaxel	0.95 +/- 0.19	0.76 +/- 0.21	0.66 +/- 0.25
Docetaxel	1.15 +/- 0.07	0.98 +/- 0.05	0.85 +/- 0.09

T47D cells		Fractional growth inhibition ^b	
Drug	0.50	0.75	0.90
Doxorubicin	1.04 +/- 0.11	0.83 +/- 0.28	0.84 +/- 0.45
c-DDP	0.95 +/- 0.13	0.75 +/- 0.07	0.67 +/- 0.13
5-FU	0.71 +/- 0.01	0.67 +/- 0.09	0.69 +/- 0.20
FdURd	0.84 +/- 0.06	0.79 +/- 0.08	0.80 +/- 0.16
4HC	1.01 +/- 0.13	0.73 +/- 0.02	0.58 +/- 0.05
Vinorelbine	1.02 +/- 0.19	0.69 +/- 0.13	0.55 +/- 0.20
Paclitaxel	0.89 +/- 0.05	0.75 +/- 0.07	0.71 +/- 0.16
Docetaxel	0.91 +/- 0.24	0.79 +/- 0.18	0.80 +/- 0.17

^a Cells were treated with drug alone on day 0 for 24 hours then the media was removed and the cell monolayer washed with drug- and serum-free media, then media with CPENSp^m was then added for the remainder of the culture period (120 hours).

^b Combination Index (CI) values for fractional growth inhibitions of 0.50, 0.75, and 0.90 in the MDA-MB-231, Hs578t, and T47D cell line. Antagonism CI > 1.00, additivity CI = 1.00, synergy CI < 1.00 +/- standard deviation.

Table 7 Effects of CHENSpm and 5-FU or FdURd on MDA-MB-231, Hs578t, and T47D cells^a

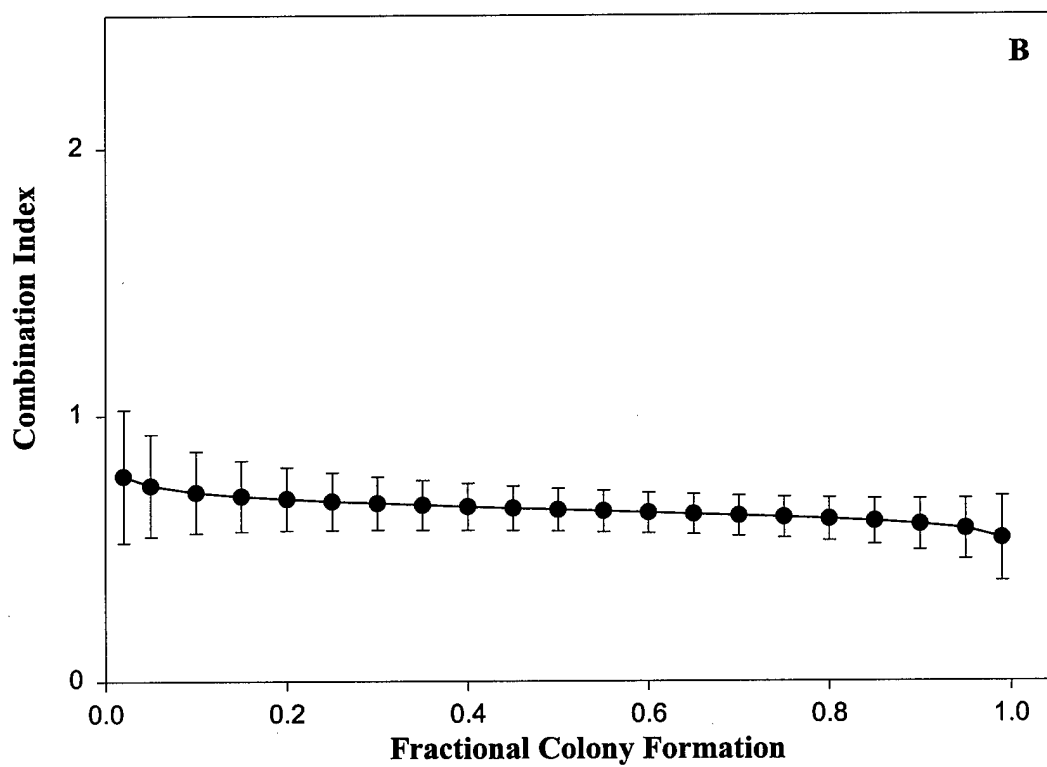
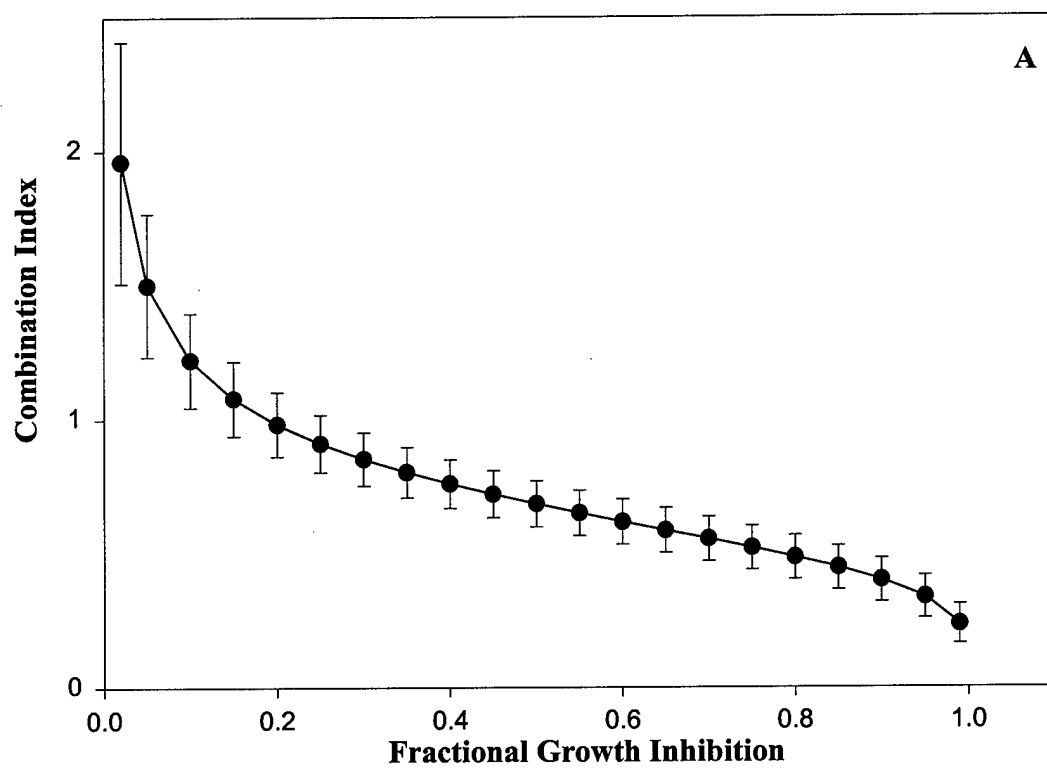
MDA-MB-231 cells		Fractional growth inhibition ^b	
Drug	0.50	0.75	0.90
5-FU	0.84	0.74	0.73
FdURd	0.56 +/- 0.06	0.54 +/- 0.21	0.67 +/- 0.36
Hs578t cells			
Drug	0.50	0.75	0.90
5-FU	0.96 +/- 0.06	0.88 +/- 0.05	0.81 +/- 0.07
FdURd	0.92 +/- 0.11	0.75 +/- 0.09	0.75 +/- 0.08
T47D cells			
Drug	0.50	0.75	0.90
5-FU	0.88 +/- 0.04	0.70 +/- 0.05	0.63 +/- 0.02
FdURd	1.04 +/- 0.06	1.02 +/- 0.12	1.20 +/- 0.27

^a Cells were treated with 5-FU or FdURd alone on day 0 for 24 hours then the media was removed and the cell monolayer washed with drug- and serum-free media, then media with CHENSpm was then added for the remainder of the culture period (120 hours).

^b Combination Index (CI) values for fractional growth inhibitions of 0.50, 0.75, and 0.90 in the MDA-MB-231, Hs578t, and T47D cell lines. Antagonism CI > 1.00, additivity CI = 1.00, synergy CI < 1.00 +/- standard deviation.

Figure legend:

Figure 1. Combination Index (CI) plotted as a function of growth inhibition (A) and clonogenic ability (B) following combination therapy using FdURd and CPENSpm in MCF-7 Cells. Cells were exposed to 24 hours of FdURd alone, washed then exposed to CPENSpm alone for the remainder of the culture period (96 hours).



Delayed Micromolar Elevation in Intracellular Calcium Precedes Induction of Apoptosis in Thapsigargin-treated Breast Cancer Cells¹

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ABSTRACT

Thapsigargin (TG), a highly specific inhibitor of the sarcoplasmic reticulum and endoplasmic reticulum Ca^{2+} -ATPase pump, can induce apoptosis in a variety of epithelial and lymphoid cell types. In prostate cancer cell lines, TG induces an initial 5- to 10-fold elevation of intracellular calcium ($[\text{Ca}^{2+}]_i$) within a few minutes of exposure. With prolonged exposure times (*i.e.*, 12–36 h) a second elevation of $[\text{Ca}^{2+}]_i$ to $>10 \mu\text{M}$ is observed. In this study, the human breast carcinoma cell lines MCF-7 and MDA MB 468 cells were used to determine the temporal relationship between TG-induced elevation of $[\text{Ca}^{2+}]_i$ and activation of programmed cell death. Using a microinjection method that allows for long-term analysis of $[\text{Ca}^{2+}]_i$ changes, we found that after TG exposure, calcium measurements in these cells demonstrated an initial rise (>4 -fold) in $[\text{Ca}^{2+}]_i$ that occurred within minutes and returned to baseline within a few hours. With prolonged TG exposure, the cells underwent a second elevation ($>5 \mu\text{M}$) of $[\text{Ca}^{2+}]_i$, occurring stochastically between 12 and 36 h after the initial exposure to TG. Both of

the cell lines were growth-inhibited by 100 nM TG after only 1 h of exposure, but clonogenic ability in the MCF-7 cells was significantly reduced only after 48 h of exposure. The induction of apoptosis by TG was demonstrated by morphological changes typical for programmed cell death and DNA fragmentation (both high molecular weight and oligonucleosomal-sized fragments were detected) after 48 h of treatment. TG induction of apoptosis in these breast cancer cells occurred subsequent to the secondary rise in $[\text{Ca}^{2+}]_i$, which confirmed that this secondary rise in $[\text{Ca}^{2+}]_i$ is not prostate cancer-specific. The secondary rise in $[\text{Ca}^{2+}]_i$ to micromolar levels may directly activate the endonucleases responsible for DNA fragmentation that occurs as part of the apoptotic process. These studies indicate that TG is an active agent *in vitro* against breast cancer cells. Inactive prodrug analogues of TG are currently being developed that can be activated by tissue-specific proteases, and further pursuit of this strategy as a potential treatment for breast cancer is warranted.

INTRODUCTION

Breast cancer is the second leading cause of cancer deaths in American women (1). Currently there is no curative therapy for metastatic breast cancer. Although many active cytotoxic agents are used in the treatment of this disease, their use is limited by inherent or acquired tumor cell drug resistance. In addition, these cytotoxic agents are also associated with often severe, dose-limiting, systemic toxicities. Therefore, the need for development of novel therapeutic agents active against breast cancer remains an important goal.

An example of such an agent is TG,⁷ a sesquiterpene- γ -lactone extracted from the seeds and roots of the umbelliferous plant, *Thapsia garganica*. TG selectively inhibits the SERCA pump (2). TG-induced inhibition of the SERCA pump leads to initial depletion of the ER Ca^{2+} pool. This depletion results in the generation of a soluble signal, possibly from an ER-derived diffusible messenger that activates plasma membrane calcium channels, thereby resulting in an influx of extracellular calcium (2). In cells exposed to TG, an initial increase in cytoplasmic Ca from a baseline of 20–50 nM to a $[\text{Ca}^{2+}]_i$ of 200–400 nM is observed within minutes after exposure to TG (3). Using prostate cancer cell lines, Tombal *et al.* (4) have demonstrated that, after the initial rise, $[\text{Ca}^{2+}]_i$ then returns to baseline. However, with prolonged exposure to TG, prostate cancer cells then undergo a second elevation of $[\text{Ca}^{2+}]_i$ to micromolar concentration

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⁷ The abbreviations used are: TG, thapsigargin; ER, endoplasmic reticulum; $[\text{Ca}^{2+}]_i$, intracellular calcium; SERCA, sarcoplasmic reticulum and ER Ca^{2+} -ATPase; HMW, high molecular weight; PCD, programmed cell death; PSA, prostate-specific antigen.

(4). This second rise occurs stochastically between 12 and 36 h after initial exposure (4) and may directly activate the endonucleases responsible for the DNA fragmentation observed as part of the apoptotic process (5).

Prolonged exposure to TG induces apoptosis in a variety of rapidly proliferating cell types *in vitro*, including breast cancers (3, 6–8). However, the majority of cells in human cancers are proliferatively quiescent, and this may in part explain the relative ineffectiveness of antiproliferative therapies. Using proliferatively quiescent, G₀-arrested, primary cultures of prostate cancers, Lin *et al.* (9) demonstrated that TG was capable of inducing proliferation-independent cell death, but the antiproliferative agent, 5-fluorouracil, had no effect.

Although TG is highly effective in inducing proliferation-independent apoptosis, it is sparingly water-soluble, its cytotoxicity is not cell-type specific, and it is able to kill proliferatively quiescent G₀ cells. For these reasons, unmodified TG would be difficult to administer and deliver systemically without significant nonspecific host toxicity. One possible strategy to circumvent the nonspecific cytotoxicity and improve the therapeutic index would be to create an inactive prodrug form of TG that can be specifically activated by breast cancer cells. Previously, Christensen *et al.* (10) have synthesized and characterized several TG analogues in which a primary amine was introduced into the TG molecule to allow the analogue to be coupled with the COOH terminus of a peptide. Using this approach, the inactive peptide-TG prodrug can be targeted for activation specifically by tissue or cancer-specific proteases, thereby avoiding systemic toxicity. Ideally, expression or activity of the protease would be restricted to the cell type being targeted.

An example of such a tissue-restricted protease is PSA. A series of studies have documented the expression of PSA by breast cancer tissue (11, 12). PSA is a M_r 33,000 serine protease initially thought to be secreted exclusively by normal and malignant prostate epithelial cells (13). However, human breast cancer cell lines can produce small amounts of PSA after incubation with steroid hormones, and production is only observed in cell lines that possess steroid hormone receptors (14). In addition, PSA immunoreactivity is detectable in about 30% of primary breast cancer cytosolic extracts (11). The presence of detectable PSA in breast cancers may be a favorable prognostic indicator, perhaps because it may serve as a marker of hormone responsiveness (15, 16). In women, significant expression of PSA seems to be restricted to breast tissue alone (17). PSA expression, however, is not restricted to breast cancer but has also been detected in breast milk, breast cyst fluid, nipple aspirates, and normal breast tissue (12, 18).

In the present study, we have measured the time course and magnitude of calcium changes in breast cancer cells after TG exposure to determine whether the late secondary rise in [Ca²⁺]_i, previously observed in prostate cells and linked to activation of PCD, also occurs in breast cancer cells. In addition, we have investigated the dose responsiveness of breast cancer cell lines to TG and examined the kinetics of cell death in TG-treated cells to determine the feasibility of targeting TG as breast cancer therapy.

MATERIALS AND METHODS

TG was obtained from Calbiochem (San Diego, CA). A 1-mM stock solution in ethanol was stored at –20°C and diluted as needed in medium for desired final concentrations. Ethanol was used for vehicle control-treated cultures. Fura 2-AM, M_r 10,000 Fura-dextran, and Fura-2 free acid were purchased from Molecular Probes (Eugene, OR). To prepare stock solutions, AM derivatives were dissolved at a 1-mM final concentration in DMSO/Pluronic F-127 (Molecular Probes). All of the other reagents were from Sigma Chemical (St. Louis, MO).

Cell Lines. MDA MB 468 and MCF 7 breast cancer cells were maintained in IMEM and DMEM, respectively, supplemented with 5% fetal bovine serum (Biofluids, Rockville, MD) and 2 mM glutamine. The MCF-7 cell line was obtained from the laboratory of Dr. Marc Lippman (Vincent Lombardi Cancer Center, Washington, DC) and the MDA-MB-468 cell line from the American Type Culture Collection (Rockville, MD). Cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere and passaged every 5 days. *Mycoplasma* testing was routinely negative.

Colony-forming Assay. MCF 7 cells were initially plated in 24-well plates at a density of 5×10^4 cells per well and allowed to attach overnight. The medium was removed and TG at 100 nM or ethanol as vehicle control in media was added to the wells for 1, 4, 24, or 48 h. The cells were counted by Coulter Counter (Hialeah, FL) and were immediately replated in drug-free, serum-containing medium in 60-mm tissue culture dishes at 200 cells per plate, a density associated with a 73% cloning efficiency in controls. After 1 week, the plates were stained with crystal violet [0.5% crystal violet in a 3:1 (v/v) mixture of distilled water:methanol], and colonies were counted. The loss of clonogenic survival was determined from the ratio of colonies in the treated cell group:colonies in the control group. All of the experiments were done in triplicate and performed at least twice.

Growth-inhibition Assay. Exponentially growing MCF-7 or MDA-MB-468 cells were plated in triplicate at $1-5 \times 10^4$ cells/cm² in 24-well plates. After attachment, the medium was changed, and cells were incubated in medium with either ethanol vehicle control or TG. After exposure to TG, the medium was removed and replaced with drug-free medium. After five days (*i.e.*, 120 h) cells were counted using a Coulter Counter. IC₅₀ determinations were made from plots of the percentage of untreated control cell number *versus* the logarithm of the drug concentration. All of the experiments were carried out at least twice, and the values reported are the mean \pm SD of individual points from all of the experiments.

Assessment of Morphology. Exponentially growing MCF-7 or MDA-MB-468 cells were incubated in the presence or absence of 100 nM TG for 48 h in 2-well Lab-tek chamber slides (Nunc, Naperville, IL). The cells were fixed with methanol, stained with 0.1 mg/ml Hoechst dye (Sigma Chemical Co.), and visualized by fluorescence microscopy with a ZEISS axioskope microscope (ZEISS, Hanover, MD) as previously described (19). Apoptotic index was defined as the percentage of apoptotic cells (number of apoptotic cells \div total number of cells \times 100%). Results were obtained from evaluation of 5000 cells per treatment group, and experiments were repeated three times.

DNA Fragmentation Assays. Exponentially growing cells were plated at a density of 1×10^4 cells/cm² and 4×10^4 cells/cm² for wild-type MCF-7 and MDA-MB-468 cells, respectively. After attachment overnight, the cells were incubated with or without TG for the desired exposure time. Analysis for oligonucleosomal DNA fragmentation was performed as described previously (20). For the analysis of HMW DNA fragmentation, the cells were resuspended at a density of $3-5 \times 10^5$ cells in low-melting-point agarose plugs and were processed as described previously (19, 20).

Determination of $[Ca^{2+}]_i$ Concentration. $[Ca^{2+}]_i$ concentrations were determined according to the previously validated method of Tombal *et al.* (4). Briefly, for longitudinal $[Ca^{2+}]_i$ determinations, MCF-7 cells were plated on cover-glassed incubation chambers coated with CellTak (Collaborative Research, Bedford, MA). Cells were then microinjected with M_r 10,000 Fura dextran (Molecular Probes, Eugene OR). Dynamic $[Ca^{2+}]_i$ measurements were performed by serial acquisition of fluorescent (emission, 510 nm) images of cells alternately excited at 340 or 380 nm. Images were also captured at 357 nm, the calcium-insensitive wavelength, to determine total intracellular concentration of Fura-dextran. Individual images were acquired at each UV wavelength with a Princeton CCD-1300-v Camera (Princeton Instruments, Trenton, NJ) and analyzed with MetaFluor software (Universal Imaging Corporation, West Chester, PA). $[Ca^{2+}]_i$ maps for individual cells were calculated after pixel-by-pixel computerized ratiometric reconstruction of cells alternately excited at 340 and 380 nm. Ratio to $[Ca^{2+}]_i$ transformation was performed using the Grynkiewicz equation (21).

Microinjected cells were first exposed to varying concentrations of TG (*i.e.*, 50–1000 nM) to determine the magnitude of the initial (*i.e.*, minutes) elevation in $[Ca^{2+}]_i$. No significant difference in the magnitude of the calcium elevation at these varying concentrations was observed (data not shown). For the delayed elevation determinations, a saturating concentration of 1 μ M TG (*i.e.*, 100-fold above IC₅₀ for SERCA inhibition) reproducibly produced a delayed elevation in >50% of injected cells within 24–36 h. Therefore, this concentration of TG was chosen to determine the time course and magnitude of $[Ca^{2+}]_i$ changes in TG-exposed cells.

Alternately, MCF-7 cells were passively loaded with 7.5 μ M for 30 min at 22°C with Fura 2-AM, and $[Ca^{2+}]_i$ were determined as described above (4).

RESULTS

TG-induced Changes in $[Ca^{2+}]_i$. The most commonly used method to determine changes in $[Ca^{2+}]_i$ has been to passively load cells with the fluorescent calcium indicator Fura 2-AM. This dye readily enters cells and must be completely de-esterified by cytoplasmic esterases to produce a negatively charged fluorescent Ca^{2+} -sensitive compound. Because of its charge, de-esterified Fura-2 is inhibited from crossing the plasma membrane and, thus, is initially concentrated in the cytoplasm. If the ester bonds are not cleaved at a sufficient rate in the cytoplasm, however, partially de-esterified Fura 2-AM can cross membranes of intracellular organelles (*i.e.*, mitochondria, ER). These organelles also contain esterase activity and

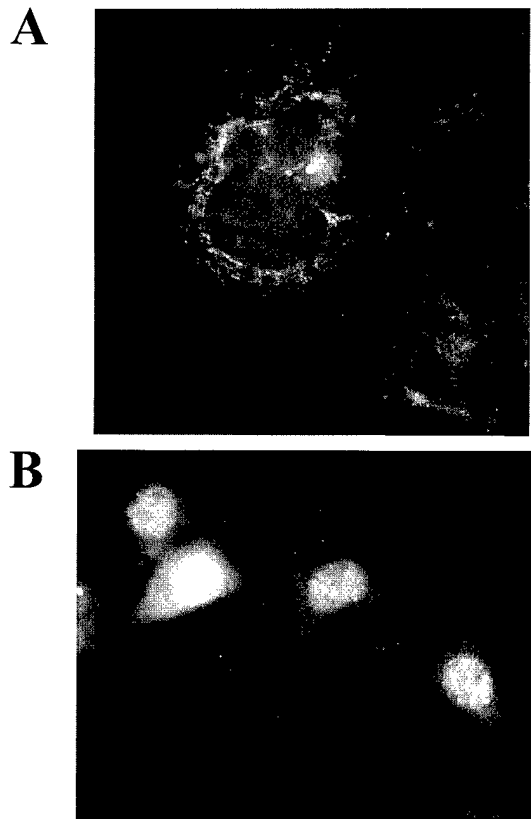


Fig. 1 A, MCF-7 cells passively loaded with 7.5 μ M Fura 2-AM at 22°C for 30 min demonstrate heterogeneous distribution of Fura dye intracellularly. Fura dye is compartmentalized into the ER and not into mitochondria as demonstrated by coincubation with 5 μ M JC-1, a mitochondrial selective probe (data not shown); B, MCF-7 cells microinjected with M_r 10,000 Fura dextran demonstrating homogeneous distribution of dye into cytoplasm and nucleus. (Images acquired using $\times 64$ objective, excitation 357 nm, and emission 510 nm).

can complete de-esterification of Fura 2-AM, trapping it within these organelles. This degree of compartmentalization is dependent on loading conditions and cell type. Compartmentalization, if not recognized, can produce spurious results when attempting to measure $[Ca^{2+}]_i$.

When MCF-7 cells are passively loaded with Fura 2-AM at 22°C, the dye rapidly compartmentalizes into the ER, Fig. 1A. Coloaded cells with rhodamine-123, a dye that is mitochondria-specific in its uptake, demonstrates that Fura-2 is only detectable in the ER and does not compartmentalize into mitochondria (data not shown). Because TG induces a rapid decrease in the ER calcium concentration, using passive loading of Fura-2 to measure $[Ca^{2+}]_i$ changes after TG exposure markedly underestimates the true magnitude of the TG-induced $[Ca^{2+}]_i$ rise.

To overcome these limitations, a microinjection method was developed in which cells are injected with the Fura dye complexed to M_r 10,000 dextran. Once microinjected into cells, Fura dextran does not require enzymatic modification to equilibrate with $[Ca^{2+}]_i$, and its HMW makes it resistant to leakage and compartmentalization (Fig. 1B). In addition, use of the M_r

10,000 Fura dextran allows for longitudinal analysis of $[Ca^{2+}]_i$ for several days, whereas $[Ca^{2+}]_i$ can be monitored for <2 h using the passive loading technique (3).

Using this validated microinjection technique, MCF-7 cells were injected with dye and exposed to TG. Analysis of initial $[Ca^{2+}]_i$ changes induced by TG in these microinjected cells demonstrates a >4-fold elevation of $[Ca^{2+}]_i$ within the first 10 min of TG exposure (Fig. 1C). In contrast, when the passive dye loading technique was used, a minimal (*i.e.*, 10%) change in $[Ca^{2+}]_i$ was noted (Fig. 1C). The >4-fold rise in $[Ca^{2+}]_i$ observed using the microinjection technique is similar in magnitude to $[Ca^{2+}]_i$ changes seen in similarly microinjected prostate cancer cell lines. In addition, in prostate cancer cell lines in which extensive dye compartmentalization is not observed, passive loading of Fura dye also resulted in detection of 4- to 7-fold elevation in $[Ca^{2+}]_i$ within minutes after TG exposure.

Although the ability to accurately detect $[Ca^{2+}]_i$ changes after initial TG exposure is useful for studying TG interaction with its SERCA pump target, TG does not immediately induce apoptosis. Instead, cells exposed to TG do not begin to undergo apoptosis for several days after initial exposure. This microinjection method makes it possible to measure $[Ca^{2+}]_i$ changes at these later time points because cells are beginning to manifest morphological and biochemical changes consistent with apoptosis.

MCF-7 cells were, therefore, microinjected with M_r 10,000 Fura-dextran and exposed continuously to 1 μ M TG. After the initial $[Ca^{2+}]_i$ rise, which was maintained for several hours (*i.e.*, 2–10 h), in each cell measured, the $[Ca^{2+}]_i$ transiently returned to baseline (*i.e.*, 80 nm). After this return to baseline, a second delayed rise in $[Ca^{2+}]_i$ was detected within 12–72 h after exposure to TG. The secondary $[Ca^{2+}]_i$ occurred asynchronously within the cell population and was much larger (*i.e.*, >1 μ M) than the initial $[Ca^{2+}]_i$ elevation seen in the first few minutes after TG exposure (*i.e.*, 200–400 nm). The precise amplitude of this delayed rise cannot be accurately measured using Fura-dextran because the Ca^{2+} binding by Fura is saturated above 1 μ M concentration of Ca^{2+} . Characteristically this delayed rise is sustained for 4–8 h before major alterations in plasma membrane permeability lead to a rapid leakage of the dye from the cytoplasm, making further $[Ca^{2+}]_i$ measurements impossible.

Activity of TG against Breast Cancer Cells *in Vitro*.

MCF-7 and MDA-MB-468 cells were continuously exposed to various concentrations of TG over 120 h, and cell counts were obtained. Growth inhibition was determined from the ratio of the treated cell number:control cell number at 120 h. The concentration needed to inhibit growth of MCF-7 cells by 50% (IC_{50}) was 3 nM and for MDA-MB-468 the IC_{50} was 1 nM. Treatment of cells with 100 nM TG resulted in >95% inhibition of growth of both lines after 120 h exposure (data not shown), and, therefore, this concentration of TG was used in additional studies to determine the kinetics of apoptosis induction.

To demonstrate that the growth inhibition after TG exposure is also associated with a decrease in clonogenic survival, the colony-forming ability of MCF 7 cells after exposure to 100 nM TG for various time points was determined (Fig. 2). Exposure times of 1 and 4 h did not have any significant effect on the colony-forming ability of the MCF-7 cells. A 24-h drug exposure resulted in only a small decrease in cloning efficiency whereas 48 h reduced the cloning efficiency to less than 20%.

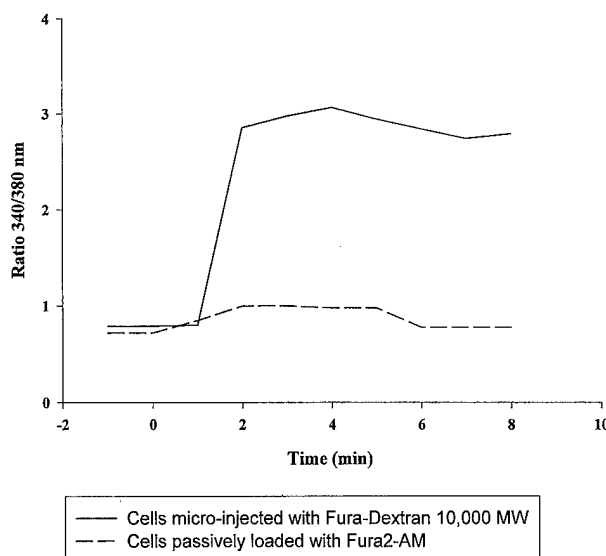


Fig. 2 Comparison of two different methods used to determine intracellular calcium changes in MCF-7 cells after exposure to 1 μ M TG. Data are given as ratio of emission at 340:emission at 380 nm. A ratio value of 0.75 corresponds approximately to 75 nm $[Ca^{2+}]_i$; a ratio value of 2.5 correspond to 400 nm $[Ca^{2+}]_i$; and a ratio value >5 is above the limit of sensitivity of the Fura-2 probe.

Despite multiple attempts under a variety of conditions, no colonies could be obtained from MDA-MB-468 cells. The 48-h time point was, therefore, used for analysis of induction of PCD by TG treatment in both of the cell lines as outlined below.

Induction of Apoptosis by TG. Morphological changes associated with PCD include nuclear condensation, chromatin aggregation, and disruption of the cell into apoptotic bodies (22). These morphological changes were observed in MCF 7 and MDA-MB-468 cells after treatment with TG. Using Hoechst staining to assess apoptotic nuclear changes, apoptotic index was low in untreated control cells after 48 h (*i.e.*, $0.45 \pm 0.07\%$ for MCF 7 cells and $2.1 \pm 0.35\%$ for MDA-MB-468 cells). In contrast, cells treated with 100 nM TG had elevated apoptotic indices at $29.3 \pm 0.39\%$ for MCF 7 cells and $25.3 \pm 1.16\%$ for MDA-MB-468 cells.

Fragmentation of genomic DNA into both HMW and oligonucleosomal-sized fragments was readily apparent in both of the cell lines after TG treatment. Fig. 3A depicts HMW DNA fragmentation in both MCF 7 and MDA MB 468 cells after exposure to 100 nM TG for 48 h. Fig. 3B shows detectable oligonucleosomal fragmentation in both of the cell lines after a 48-h exposure to 100 nM TG. Minimal or no HMW or oligonucleosomal DNA fragmentation was detected in either cell line at time zero or in untreated control cells.

Both HMW and oligonucleosomal DNA fragmentation evaluations as described above are qualitative estimates of PCD within these model systems. To better assess the amount of DNA fragmentation over time in both cell lines, we also used quantitative assay for DNA fragmentation. [^{14}C]thymidine-labeled cells were subjected to continuous exposure to 100 nM TG. Release of radiolabel into the culture media and the amount incorporated into HMW DNA fragments (as described in the

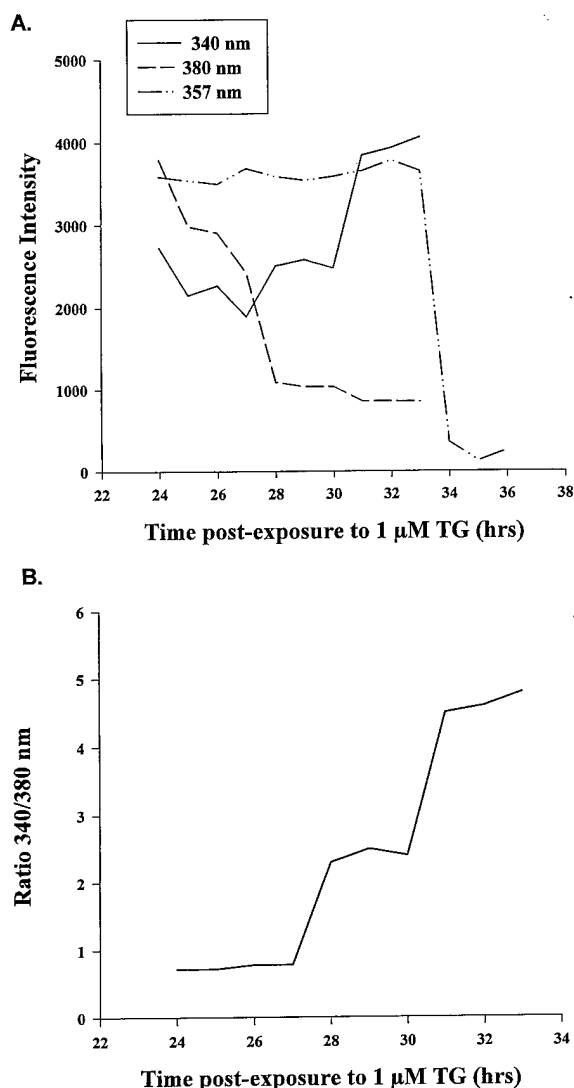


Fig. 3 A trace of the delayed second elevation in intracellular calcium observed in a single MCF-7 cell after exposure to 1 μ M TG. A total of 12 cells were analyzed and a representative trace is shown. A, fluorescence intensity at 340 nm (calcium-bound Fura-dye), 380 nm (unbound Fura dye), and 357 nm (calcium binding insensitive). The fluorescence intensity at 357 nm demonstrates that the increase in the ratio of emission at 340 nm:emission at 380 nm is not attributable to leakage of the M_f 10,000 Fura-dextran out of the cell. Dye leakage does occur (as depicted by the decrease in 357-nm intensity) 4–6 h after the onset of the second calcium rise. B, increase in the ratio of emission at 340 nm:emission at 380 nm from that in A. The increasing ratio is proportional to the increasing intracellular calcium as described in Fig. 2.

“Materials and Methods”) were measured at several time points over a 120-h culture period. The results of these experiments are shown in Figs. 4, 5, and 6. MCF-7 cells show an increase in the percentage of DNA fragmentation over control by 48 h (Fig. 5) with a continued rise observed over the remainder of the culture period (120 h) (Fig. 6). Untreated control cells demonstrate a static level of DNA fragmentation over the same time period. The MDA-MB-468 breast cancer cell line also shows a steady

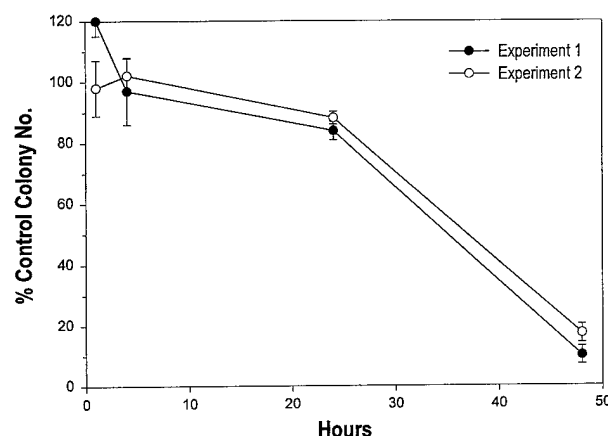


Fig. 4 Clonogenic survival of MCF-7 cells exposed to 100 nM TG. An equal number of cells (*i.e.*, 200) were seeded after exposure to TG for the indicated times. After 1 week, plates were stained, and colonies were counted. Data shown are ratios of treated colonies:control colonies (*i.e.*, percentage of control colony number) at indicated TG-exposure times.

increase in the percentage of DNA fragmentation (compared with control cells) over time beginning at 48 h (Fig. 5). The untreated control MDA-MB-468 cells demonstrate only a small increase in DNA fragmentation over the time period evaluated. The maximal absolute amount of DNA fragmentation (treated minus untreated control) is approximately 40–50% for both cell lines after 120 h in TG-containing media (Fig. 6).

DISCUSSION

Targeted antitumor prodrug therapy strategies are attractive in that an active agent can be delivered to tumor cells in a dose-intensive and -selective manner, thereby maximizing efficacy and minimizing toxicity. In this study, the inactive prodrug would consist of an analogue of TG coupled with a protease-specific peptide carrier such that the peptide-TG bond is a hydrolyzable only by the specific protease produced within the extracellular fluid of the tumor (20). TG released into the extracellular fluid would be toxic to both protease producing and nonproducing cells, (*i.e.*, “the bystander effect”). In addition, Lewalle *et al.* (23) have observed an elevation in intracellular calcium in HUVEC cells after contact with MCF-7 cells. TG pretreatment blocked this calcium elevation and disrupted endothelial cell migration *in vitro* (23). These results suggest that release of the TG analogue into the tumor microenvironment may also have an antiangiogenic effect that would disrupt interactions between tumor epithelial cells and vascular endothelial cells.

A potential breast tissue-specific protease that can be targeted in this manner is PSA, a protease produced in approximately one-third of breast cancers but not in other tissues in women (11). Cleavage of TG from the peptide carrier would deliver active drug at the site of PSA production, while minimizing systemic exposure to active drug. Previously, Denmeade *et al.* (24, 25) have identified a six-amino-acid peptide with the amino acid sequence His-Ser-Ser-Lys-Leu-Gln, which is specifically and efficiently hydrolyzed by PSA. A TG analogue can be coupled to this peptide and tested for activation by PSA-

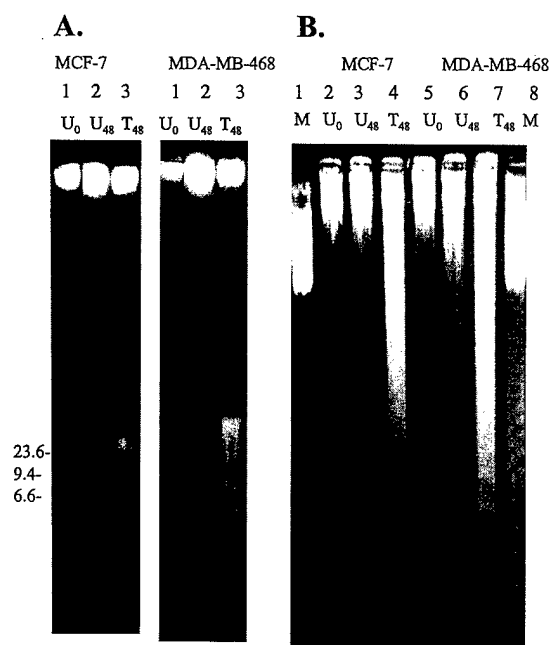


Fig. 5 DNA fragmentation resulting from TG exposure of MCF-7 and MDA-MB-468 cells. **A**, breast cancer cells were incubated in the presence or absence of TG (100 nM) for 48 h, then analyzed by field inversion gel electrophoresis to assess HMW DNA fragmentation. *Lane 1*, untreated cells at time zero; *Lane 2*, untreated cells at 48 h; *Lane 3*, is treated cells at 48 h. The numbers at left, the position of α HindIII markers. **B**, oligonucleosomal DNA fragmentation in the cell lines treated with TG as described above. *Lanes 1* and *8*, 123-bp DNA ladder marker; *Lanes 2* and *5*, untreated cells at time zero for MCF-7 and MDA-MB-468 cells, respectively; *Lanes 3* and *6*, untreated MCF-7 and MDA-MB-468 cells, respectively, at 48 h; *Lanes 4* and *7*, TG-treated MCF-7 and MDA-MB-468 cells, respectively, at 48 h.

producing breast cancer cells as potential targeted therapy for metastatic breast cancer.

Although PSA may be a potential target in 30% of breast cancers, other more highly expressed breast cancer-specific proteases may ultimately be more appropriate therapeutic targets. Breast cancer cells do in fact express a variety of proteases. Examples include cathepsins B, D, and L and the matrix metalloproteinases (26–28). The expression of these proteases by breast cancers may have prognostic significance, but their expression is not restricted to breast tissue alone. Thus, they are not likely to be useful in this strategy. Recently, the expression of two new serine proteases, matriptase (29) and protease M (30), have been described. The breast tissue specificity of these two new proteases and degree of expression by malignant breast epithelial cells has yet to be fully characterized; if differential expression is found, then these proteases may be potential candidates for prodrug targeting.

In both the MCF-7 and MDA-MB-468 cells, 1-h treatment with 100 nM TG concentration was sufficient to produce a 60–80% decrease in cell number *versus* control. However, a decrease in the clonogenic survival was not detectable in MCF-7 cells until 24 h of exposure. Previously, Furuya *et al.* (3) demonstrated that TG could cause growth-arrest of rapidly proliferating prostate cancer cell lines. In addition, Lin *et al.* (9) demonstrated that when using the rat

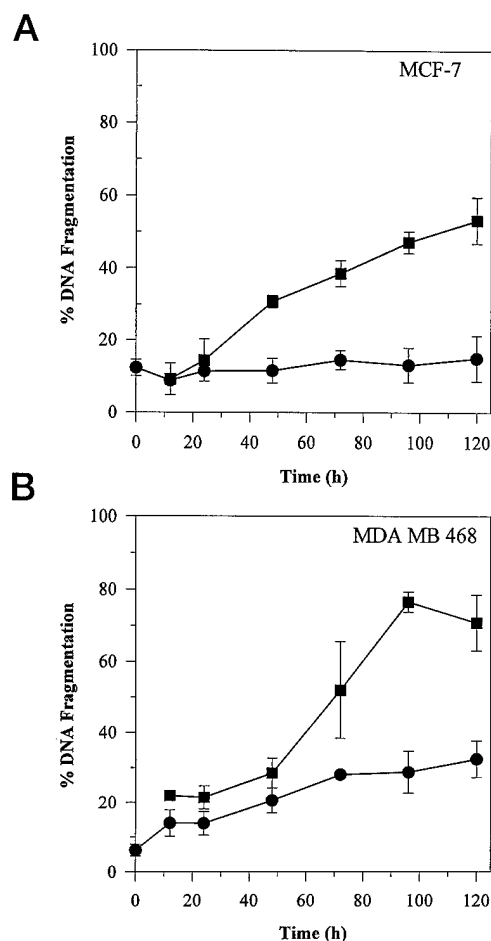


Fig. 6 Percentage of DNA fragmentation during continuous exposure to 100 nM TG or vehicle control over a 120-h exposure period. **A**, MCF-7; **B**, MDA-MB-468. ■, TG-treated cells; ●, vehicle-treated controls.

prostate cancer cell line AT3-1, TG induced growth-arrest within the first 24 h of TG exposure. In this study, TG treatment resulted in rapid increase in expression of a gene associated with growth-arrest and DNA damage (*gadd*) called *gadd153* (9). *gadd153* gene expression is known to be regulated by elevation in $[Ca^{2+}]_i$ and Lin *et al.* demonstrated that *gadd153* mRNA levels increased after 1 h of TG treatment (9). More than 24 h of exposure, however, were needed to irreversibly commit AT3-1 cells to undergo DNA fragmentation and subsequent cellular fragmentation into apoptotic bodies (9).

In the present study, a decrease in MCF-7 cell number relative to control cells was also observed with short-term exposure to TG (*i.e.*, 60% after 1 h and 90% after 24 h of exposure to 100 nM TG; data not shown). These short exposure times, however, were not sufficient to irreversibly commit MCF-7 cells to undergo apoptosis, as evidenced by the lack of significant loss of clonogenic ability in cells exposed to TG for less than 24 h. These results emphasize the pitfalls of using growth inhibition as the end point in assessing drug toxicity, because such analysis does not differentiate cytostatic from cytotoxic effects. In addition, these results provide important

information about the kinetics of TG- induced cell death. The data suggest that prolonged exposure of the target cells to TG will be necessary for the TG-peptide prodrug therapy to be effective. Therefore, prodrug administration by continuous infusion may prove to be the preferred method of drug delivery.

In this study, ~75% of cells underwent this delayed micromolar rise in $[Ca^{2+}]_i$ by 36 h of exposure to 1 μ M TG. This delayed secondary rise in $[Ca^{2+}]_i$ observed in TG-treated MCF-7 cells temporally precedes the onset of apoptotic morphological changes and DNA fragmentation and the loss of clonogenic ability. These findings suggest a causal relationship between the delayed rise in $[Ca^{2+}]_i$ and the activation of the apoptotic process. In addition, these studies confirm the findings of Tombal *et al.* (4) and demonstrate that the delayed $[Ca^{2+}]_i$ rise after TG exposure is not prostate cell-specific but, instead, may be generalizable to multiple cell types. Tombal *et al.* describe similar delayed $[Ca^{2+}]_i$ elevation in prostate cancer cells after exposure to such diverse agents as 5-fluorouracil, ionizing radiation, doxorubicin, or transforming growth factor β -1 (4). Additional studies are needed to determine whether similar $[Ca^{2+}]_i$ changes are seen in breast cancer cells after exposure to cytotoxic agents. Studies are currently underway to understand the biochemical and epigenetic changes underlying these $[Ca^{2+}]_i$ changes to determine whether these late $[Ca^{2+}]_i$ alterations are critical in the reversible "triggering" or signaling phase of apoptosis or are merely an additional component of the irreversible, "killing" or execution phase.

REFERENCES

- Landis, S. H., Murray, T., Bolden, S., and Wingo, P. A. Cancer statistics, 1999. *CA Cancer J. Clin.*, 49: 8–31, 1999.
- Thastrup, O., Cullen, P. J., Drøbak, B. K., Hanley, M. R., and Dawson, A. P. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} -ATPase. *Proc. Natl. Acad. Sci., USA*, 87: 2466–2470, 1990.
- Furuya, Y., Lundmo, P., Short, A., Gill, D., and Isaacs, J. T. The role of calcium, pH, and cell proliferation in the programmed (apoptotic) death of androgen-independent prostatic cancer cells induced by thapsigargin. *Cancer Res.*, 54: 6167–6175, 1994.
- Tombal, B., Denmeade, S. R., and Isaacs, J. T. Assessment and validation of a microinjection method for kinetic analysis of $[Ca^{2+}]_i$ in individual cells undergoing apoptosis. *Cell Calcium*, 1: 19–28, 1999.
- Tombal, B., Weeraratna, A. T., Denmeade, S. R., and Isaacs, J. T. Thapsigargin induces a calmodulin/calcineurin-dependent apoptotic cascade responsible for the death of prostatic cancer cells. *Prostate*, in press, 2000.
- Qi, X. M., He, F., Zhong, H., and Distelhorst, C. W. Baculovirus p35 and Z-VAD-fmk inhibit thapsigargin-induced apoptosis of breast cancer cells. *Oncogene*, 15: 1207–1212, 1997.
- McColl, K. S., He, H., Zhong, H., Whitacre, C. M., Berger, N. A., and Distelhorst, C. W. Apoptosis induction by the glucocorticoid hormone dexamethasone and the calcium-ATPase inhibitor thapsigargin involves Bcl-2 regulated caspase activation. *Mol. Cell. Endocrinol.*, 139: 229–238, 1998.
- Takadera, T., and Ohayashiki, T. Apoptotic cell death and CPP32-like activation induced by thapsigargin and their prevention by nerve growth factor in PC12 cells. *Biochim. Biophys. Acta*, 1401: 63–71, 1998.
- Lin, X. S., Denmeade, S. R., Cisek, L., and Isaacs, J. T. The role of growth arrest in programmed (apoptotic) death of prostate cancer cells by thapsigargin. *Prostate*, 33: 201–207, 1997.
- Christensen, S. B., Andersen, A., Kromann, H., Treiman, M., Tombal, B., Denmeade, S. R., and Isaacs, J. T. Thapsigargin analogues for targeting programmed death of androgen-independent prostatic cancer cells. *Bioorg. Med. Chem.*, 7: 1273–1280, 1999.
- Diamandis, E. P., Yu, H., and Sutherland, D. J. A. Detection of prostate-specific antigen immunoreactivity in breast tumors. *Breast Cancer Res. Treat.*, 32: 301–310, 1994.
- Yu, H., Diamandis, E. P., Levesque, M., Giai, M., Roagna, R., Ponzzone, R., Simondi, P., Monne, M., and Croce, C. Prostate specific antigen in breast cancer, benign breast disease and normal breast tissue. *Breast Cancer Res. Treat.*, 40: 171–178, 1996.
- Wang, M. C., Valenzuela, L. A., Murphy, G. P., and Chu, T. M. Purification of a human prostate specific antigen. *Investig. Urol.*, 17: 159–163, 1979.
- Zarghami, N., Grass, L., and Diamandis, E. P. Steroid hormone regulation of prostate-specific antigen gene expression in breast cancer. *Br. J. Cancer*, 75: 579–588, 1997.
- Yu, H., Giai, M., Diamandis, E., Katsaros, D., Sutherland, D. J. A., Levesque, M. A., Roagna, R., Ponzzone, R., and Simondi, P. Prostate-specific antigen is a new favorable prognostic indicator for women with breast cancer. *Cancer Res.*, 55: 2104–2110, 1995.
- Yu, H., Levesque, M. A., Clark, G. M., and Diamandis, E. P. Prognostic value of prostate-specific antigen for women with breast cancer: a large United States cohort study. *Clin. Cancer Res.*, 4: 1489–1497, 1998.
- Diamandis, E. P., and Yu, H. Nonprostatic sources of prostate-specific antigen. *Urol. Clin. North Am.*, 24: 275–282, 1997.
- Lai, L. C., Erbas, H., Lennard, T. W. J., and Peaston, R. T. Prostate-specific antigen in breast cyst fluid: possible role of prostate-specific antigen in hormone-dependent breast cancer. *Int. J. Cancer*, 66: 743–746, 1996.
- Furuya, Y., and Isaacs, J. T. Proliferation dependent *versus* independent programmed cell death of prostatic cancer cells involves distinct gene regulation. *Prostate*, 25: 301–309, 1994.
- Armstrong, D. K., Isaacs, J. T., Ottaviano, Y. L., and Davidson, N. E. Programmed cell death in an estrogen-independent human breast cancer cell line, MDA-MB-468. *Cancer Res.*, 52: 3418–3424, 1992.
- Gryniewicz, G., Poenie, M., and Tsien, R. Y. A new generation of Ca^{2+} indicator with greatly improved fluorescence properties. *J. Biol. Chem.*, 260: 3440–3450, 1985.
- Kerr, J. F. R., Wyllie, A. G., and Currie, A. R. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer*, 26: 239–257, 1972.
- Lewalle, J. M., Cataldo, D., Bajou, K., Lambert, C. A., and Foidart, J. M. Endothelial cell intracellular Ca^{2+} concentration is increased upon breast tumor cell contact and mediates tumor cell transendothelial migration. *Clin. Exp. Metastasis*, 16: 21–29, 1998.
- Denmeade, S. R., Lou, W., Malm, J., Lovgren, J., Lilja, H., and Isaacs, J. T. Specific and efficient peptide substrates for assaying the proteolytic activity of prostate specific antigen. *Cancer Res.*, 57: 4924–4930, 1997.
- Denmeade, S. R., Nagy, A., Gao, J., Lilja, H., Schally, A., and Isaacs, J. T. Enzymatic-activation of a doxorubicin-peptide prodrug by prostate-specific antigen. *Cancer Res.*, 58: 2537–2540, 1998.
- Xing, R., Addington, A. K., and Mason, R. W. Quantification of cathepsins B and L in cells. *Biochem. J.*, 332: 499–505, 1998.
- Foekens, J. A., Look, M. P., Bolt-de Vries, J., Meijer-van Gelder, M. E., van Putten, W. L. J., and Kilijn, J. G. M. Cathepsin-D in primary breast cancer: Prognostic evaluation involving 2810 patients. *Br. J. Cancer*, 79: 300–307, 1999.
- Dickson, R. B., Shi, Y. E., and Johnson, M. D. Matrix-degrading proteases in hormone-dependent breast cancer. *Breast Cancer Res. Treat.*, 31: 167–173, 1994.
- Lin C-Y, Wang, J. K., Torri, J., Dou, L., Sang, Q. A., and Dickson, R. B. Characterization of a novel, membrane-bound, 80-kDa matrix-degrading protease from human breast cancer cells. Monoclonal antibody production, isolation, and localization. *J. Biol. Chem.*, 272: 9147–9152, 1997.
- Anisowicz, A., Sotiropoulou, G., Stenman, G., Mok, S. C., and Sager, R. A novel protease homolog differentially expressed in breast and ovarian cancer. *Mol. Med.*, 2: 624–636, 1996.



Novobiocin in Combination With High-Dose Chemotherapy for the Treatment of Advanced Breast Cancer: A Phase 2 Study

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ABSTRACT

We conducted the first phase 2 and pharmacologic study to evaluate the combination of novobiocin (a coumeromycin antibiotic that has been shown to augment alkylating agent cytotoxicity in experimental models) and high-dose cyclophosphamide and thiotepa followed by autologous marrow support in women with chemosensitive advanced breast cancer. Its aims were (1) to determine progression-free survival (PFS) and overall survival (OS), (2) to evaluate the pharmacokinetics of cyclophosphamide and thiotepa, and (3) to measure the ability of novobiocin to reverse alkylator drug resistance in vitro. Forty-one women with chemotherapy-responsive advanced breast cancer received cyclophosphamide (4 g/m²) for peripheral blood stem cell mobilization (treatment 1) followed by high-dose cyclophosphamide (1.5 g/m² per day for 4 days), thiotepa (200 mg/m² per day for 4 days), and novobiocin (4 g/day orally for 7 days) (treatment 2) and autologous marrow support. The median PFS was 10 months (range, 0.2-70.6 months) and OS, 21.5 months (range, 0.2-70.6 months). There was no statistically significant relationship between PFS or OS and area-under-the-curve values of cyclophosphamide, thiotepa, or 4-hydroxycyclophosphamide. Patient plasma samples (n = 12) obtained during novobiocin therapy were able to reverse alkylator drug resistance in an in vitro colony-forming assay. Correlative laboratory studies in an in vitro model system demonstrated that patient plasma after novobiocin treatment resulted in the magnitude of resistance reversal that had been predicted by prior preclinical experiments. Clinically, however, this activity of novobiocin did not translate into a substantial increase in PFS or OS compared with historical controls treated with high-dose alkylator therapy alone.

KEY WORDS

Drug resistance • Alkylating agents • Dose intensity

INTRODUCTION

High-dose therapy with peripheral blood stem cell (PBSC) or bone marrow support for advanced breast cancer has resulted in high response rates, including complete response (CR) rates of >50%, but only 15% to 20% of

responding women achieve long-term disease-free survival [1-6]. These results demonstrate that this therapy is unable to overcome drug resistance in the majority of patients. Therefore, the need to circumvent drug resistance remains an important goal in the treatment of breast cancer.

Investigational approaches designed to overcome drug resistance include evaluation of new chemotherapeutic preparative regimens, use of sequential high-dose therapy, immune treatment modalities, and inclusion of agents that may reverse resistance to therapy [7-9]. Dose intensity has been shown to be important in the treatment of metastatic breast cancer [10,11], and experimental studies have demonstrated that drug resistance can be at least partially overcome by dose escalation [12]. Alkylating agents (which universally are a component of high-dose therapy) have been shown to have a steep dose-response curve with a generally linear relationship between dose and log cell kill [12,13].

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Alkylating agents induce cell death by direct DNA damage, and one possible avenue to augment their activity is inhibition of DNA repair. The coumeromycin antibiotic novobiocin has several effects, including inhibition of DNA repair, on the DNA of prokaryotic and eukaryotic cells [14,15]. Novobiocin has been shown to augment alkylating agent cytotoxicity *in vitro* and *in vivo*, whereas novobiocin treatment alone has minimal cytotoxicity [15-18]. The activity of novobiocin in combination with alkylating agents is schedule-dependent, with synergy occurring when novobiocin treatment precedes or is concomitant with alkylator administration [15,17,19].

Three clinical trials that evaluated novobiocin in combination with alkylating agents have demonstrated that novobiocin can safely be given with alkylating agent therapy [7,20,21]. We previously reported the first phase 1 study of novobiocin in combination with high-dose cyclophosphamide (CTX) and thiotepa plus PBSC/bone marrow rescue in women with metastatic or locally advanced inoperable breast cancer [7]. The maximally tolerated dose of novobiocin was 4 g/day, mucositis and vomiting being the dose-limiting toxicities. The severity of mucositis correlated with novobiocin plasma levels. Plasma levels of novobiocin >100 µg/mL were consistently observed in patients who received doses of >2g daily. These plasma levels met or exceeded *in vitro* levels that have been shown to augment cytotoxicity of the alkylators in experimental models [15-19].

This report summarizes the results of the only phase 2 clinical trial examining novobiocin in combination with high-dose CTX and thiotepa followed by autologous PBSC and bone marrow reinfusion in women with chemosensitive advanced breast cancer. The aims of the study were (1) to determine progression-free survival (PFS) and overall survival (OS), (2) to evaluate the pharmacokinetics of CTX and thiotepa and their impact on long-term disease-free survival, and (3) to measure the ability of novobiocin to reverse alkylator drug resistance in an *in vitro* assay using patient plasma samples.

PATIENTS AND METHODS

Patients

Women between the ages of 18 and 60 years with histologically documented stage IIIB or IV breast cancer, a stable complete or partial response to induction chemotherapy (as defined by standard Eastern Cooperative Oncology Group [ECOG] criteria), and ECOG performance status <2 were eligible for this study [22]. Tumor level of estrogen or progesterone receptor was not a criterion for the study. All patients had to have adequate renal, hepatic, cardiac, pulmonary, and hematopoietic reserves to undergo high-dose alkylator therapy. All patients had bone marrow biopsy results negative for tumor by routine histologic examination. Conduct of the trial was approved by the Joint Commission on Clinical Investigation of the Johns Hopkins Hospital. All patients provided written informed consent.

Bone Marrow and PBSC Harvest

All patients received 3 to 10 cycles of standard-dose outpatient chemotherapy (doxorubicin-based, $n = 37$). Marrow was collected approximately 4 to 6 weeks after initiation of the last

cycle of induction chemotherapy. A mononuclear cell preparation was obtained after density centrifugation on Ficoll-Hypaque (Biowhittaker, Walkersville, MD), incubated with 60 µg/mL of 4-hydroperoxycyclophosphamide (4HC), washed, and frozen in the liquid phase of nitrogen as described [3,23].

On day 0, the day after bone marrow harvest, patients were treated with CTX 4 g/m² intravenously (IV) (treatment 1). From day 1 to 14, patients received granulocyte-macrophage colony-stimulating factor at 5 µg/kg per day subcutaneously. On day 15, the patients underwent a single 6-hour high-flow-rate leukapheresis to obtain PBSC; the leukapheresis product was cryopreserved as described [24,25].

Novobiocin, High-Dose Chemotherapy, and Supportive Care

After blood count recovery, patients were admitted for high-dose chemotherapy. Treatment with novobiocin (Albamylin; Upjohn, Kalamazoo, MI) was initiated on day -10 at a dose of 4 g/day by mouth in 2 divided doses for a total of 14 doses. Immediately after the fourth dose of novobiocin, patients began high-dose chemotherapy of CTX 1.5 g/m² per day and thiotepa 200 mg/m² per day, both given by continuous IV infusion for 4 days from day -8 to day -5 (treatment 2). Patients received intensive IV hydration as prophylaxis against CTX-induced hemorrhagic cystitis. PBSC and purified bone marrow were infused on day -1 and 0, respectively. All patients received prophylactic antibiotic treatment with norfloxacin 400 mg orally every 12 hours and vancomycin 500 mg IV every 12 hours, both initiated on day -3 until recovery of neutrophils (neutrophil count >500/µL). Packed red blood cell transfusions were given as needed to keep the hematocrit >30%, and single-donor platelets were given as needed to keep the platelet count >20,000/µL. All blood products were irradiated to prevent graft-versus-host disease.

Evaluation of Toxicity

Patients were evaluated prospectively for toxicity with daily physical examinations, hemograms, and blood chemistries and appropriate radiographs as needed. Left ventricular ejection fraction was measured via radionuclide angiography at completion of induction chemotherapy on day 8 and after hospital discharge.

Pharmacology and Pharmacokinetics

Blood and urine specimens were collected for determination of plasma CTX, 4HC/aldophosphamide, and thiotepa concentrations and urine CTX concentrations. During both treatments, blood samples were obtained several times during and for 24 hours after CTX infusion as described [26,27]. Urine was collected up to 32 and 120 hours after infusion began for treatments 1 and 2, respectively. Plasma and urine aliquots were stored at -20°C until analysis. CTX, 4HC/aldophosphamide, and thiotepa concentrations were measured using gas chromatography as described [26-30]. Area-under-the-curve (AUC) values were calculated using the linear trapezoidal rule as described by Chiou [31]. CTX and 4HC/aldophosphamide AUC values from these patients were previously included in a larger pharmacokinetic analysis of patients with advanced breast cancer treated at our institution with high-dose chemotherapy followed by autologous bone marrow transplantation [26,27,32].

Table 1. Patient Characteristics (n = 41)

	n	%
Age range (years)		
30-39	12	29
40-49	20	49
50-59	8	20
60-69	1	2
Estrogen receptor status		
Positive	15	37
Negative*	26	63
Disease stage		
IIIB	4	10
IV	37	90
Stage IV patients		
Disease sites		
Locoregional	28	76
Bone	10	27
Visceral	19	51
Number of disease sites per patient		
1 Site		
Locoregional	14	38
Bone	0	0
Visceral	7	19
2 Sites		
Locoregional and bone	4	11
Locoregional and visceral	6	16
Bone and visceral	2	5
3 Sites		
Locoregional, bone, and visceral	4	11
Chemotherapy exposure before induction chemotherapy		
None	13	34
Adjuvant	22	58
Adjuvant and metastatic	3	8
Previous hormonal therapy		
Yes	15	39
No	23	61
Response to induction chemotherapy		
Partial	28	76
No evidence of stage IV disease before induction therapy		
Complete	2	5
	8	21

* <10 fmol/mg cytosolic protein.

Laboratory Evaluation of Plasma Novobiocin Ability to Reverse Alkylator Cytotoxicity

A clonogenic assay was used to assess the cytotoxicity of 4HC (a gift of Dr. O.M. Colvin, Duke Comprehensive Cancer Center, Durham, NC) in 2 breast cancer cell lines: a wild-type MCF 7 cell line (a gift of Dr. K. Cowan, National Cancer Institute, Bethesda, MD) and a CTX-resistant clone of the cell line CTX MCF 7 (a gift of Dr. B. Teicher, Dana-Farber Cancer Institute, Boston, MA). The CTX MCF 7 cell line was selected by weekly 1-hour pulse exposures to escalating doses of 4HC. These cells demonstrate a 9-fold resistance to 4HC at 1 log of cell kill, and resistance is stable for up to 2 months without 4HC exposure.

Plasma samples were drawn from patients immediately before the first and fourth doses of novobiocin and used as control and novobiocin plasma, respectively. The time point at which the novobiocin plasma was drawn was the same time

point at which novobiocin plasma levels (median 282 $\mu\text{g/mL}$) were measured in the phase 1 study (7). These plasma samples were separated by centrifugation, stored at -20°C , and thawed immediately before use. Cells in exponential growth were released with trypsin and suspended in these plasma samples 24 hours before, during, and 24 hours after a 1-hour exposure to varying concentrations of 4HC to determine the impact of novobiocin in the plasma on 4HC dose-response curves. After drug exposure, the cells were rinsed 3 times in phosphate-buffered saline and plated in duplicate at 3 dilutions in fresh medium with 10% fetal calf serum for colony formation. Cells were incubated for 14 days, fixed with 25% methanol, and stained with crystal violet. Colonies with >50 cells were scored visually by microscopy. The concentration of 4HC required to inhibit clonogenic growth by 90% (IC_{90}) in each cell line was calculated from these results.

Statistical Analysis

Survival was measured from the date of PBSC infusion to the date of death or analysis. PFS was defined as the interval between the date of PBSC infusion and first evidence of disease progression. Event time distributions were estimated using the method of Kaplan and Meier [33]. All results are presented as of May 7, 1999. Cox model statistical analysis was done to assess the relationship of both OS and PFS with CTX, 4HC, or thiotepa AUC values.

RESULTS

Patient Characteristics

Between March 1993 and August 1995, 43 women were registered to this study. One patient developed recurrent breast cancer after induction chemotherapy and was therefore ineligible for enrollment. A second patient manifested disease progression during therapy for PBSC mobilization and did not continue on the protocol. Forty-one women completed all protocol therapy and were available for long-term follow-up. Patient characteristics are listed in Table 1. The median age was 45 years (range, 30-61 years). Four women had stage IIIB disease, and 37 had stage IV breast cancer. The median number of recurrent disease sites (locoregional, bone, or visceral) in stage IV patients was 1. Of the patients with stage IV disease, 21 (57%) had 1 site of involvement, 12 (32%) had 2 sites, and 4 (11%) had disease in 3 sites. Prior chemotherapy (pre-induction chemotherapy) and hormonal exposure are shown in Table 1. In patients with stage IV disease, 2 of 38 (5%) had no evidence of disease (NED) before induction chemotherapy, and 8 of 38 (21%) had a CR after induction chemotherapy. In addition, 8 of the 26 women (1 patient died in hospital) with a PR after induction chemotherapy converted to CR after high-dose therapy. Median time from PBSC infusion to date of analysis was 64.9 months (range, 43.7-72.9 months) with a median follow-up of 21.5 months (range, 0.2-70.6 months).

Nine patients (2 in stage IIIB and 7 in stage IV) remained in CR at the time of analysis. Of the 7 stage IV patients free of disease, 2 were stage IV NED pre-induction chemotherapy, 4 achieved a CR with induction chemotherapy, and 1 converted to CR after high-dose therapy. The median follow-up in the group of patients who remain disease-free is 61.6 months (range, 44-70.6 months). PFS

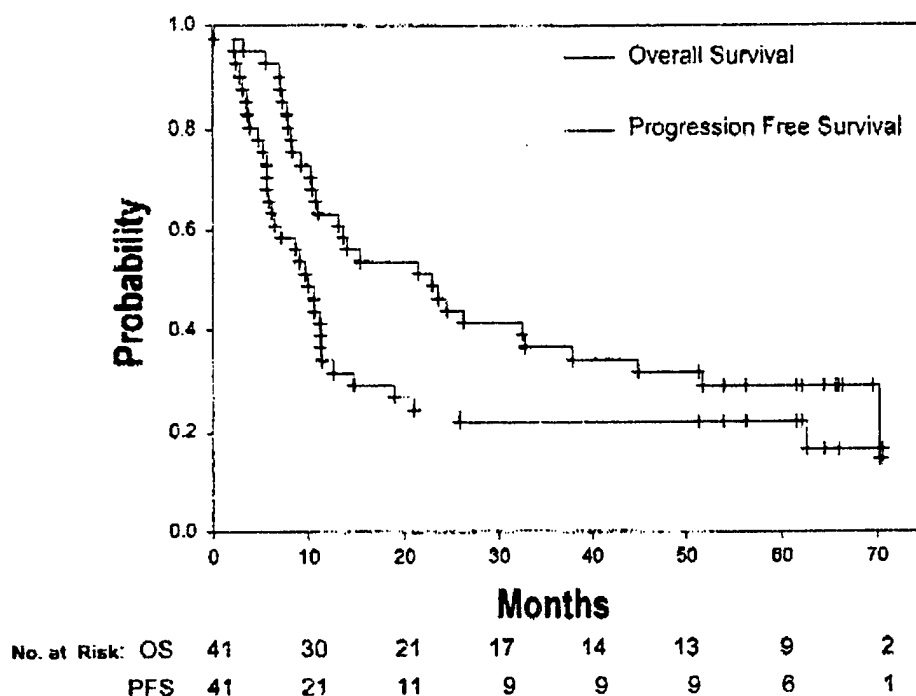


Figure 1. Results of clonogenic studies in both parental and cyclophosphamide-resistant MCF-7 breast cancer cell lines after exposure to patient plasma samples before and during novobiocin therapy.

Figure 3 text

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and OS curves are depicted in Figure 1. The median times for PFS and OS were 10 months (range, 0.2-0.6 months) and 21.5 months (range, 0.2-70.6 months), respectively.

Toxicities

Toxicities are listed in Table 2. Toxicities following CTX therapy for PBSC mobilization (treatment 1) included neutropenic fever with negative blood cultures (13 patients), acute renal failure (1 patient), and catheter-related thrombosis (1 patient). The episode of acute renal failure was completely reversible and believed to be secondary to the use of ibuprofen, which was given as part of the regimen for PBSC mobilization. No stem cell mobilization-associated toxicity prevented a patient from receiving high-dose therapy.

There was 1 therapy-related death after high-dose chemotherapy. The patient had respiratory decompensation approximately 18 hours after completion of infusional CTX and thiotepa with resultant respiratory and cardiac arrest and secondary anoxic brain damage. The patient was maintained on a respirator with maximal support (including reinfusion of PBSC) for 1 week. The patient was then extubated and died 6 days after PBSC reinfusion. No autopsy was performed. Unfortunately, this patient did not have pharmacokinetic determination to assess how drug disposition may have contributed to her outcome.

After high-dose therapy, aplasia and fever were universal, and all patients were given empiric broad-spectrum antibiotics. Infectious complications included 5 positive blood cultures, 3 episodes of oropharyngeal herpes simplex viral infection, 1 fungal pneumonia (diagnosed by characteristic computed tomography scan findings and clinical response to amphotericin B therapy), and 1 dermatomal

varicella zoster reactivation. Additional toxicity included 1 episode of hemorrhagic cystitis.

Rehospitalizations not related to recurrent disease occurred in 6 patients. Indications for readmission included catheter-related infection ($n = 2$), systemic varicella infection ($n = 1$), congestive heart failure ($n = 1$), and a severe desquamative rash related to sulfamethoxazole/trimethoprim ($n = 1$).

Pharmacokinetic Analysis

Blood samples for determination of plasma CTX concentrations during treatments 1 and 2 and thiotepa levels during treatment 2 were available in 35 (85%) of the 41 patients. Plasma 4HC/aldophosphamide measurements for both treatments were obtained in 10 (24%) patients. Urine CTX concentrations were determined in 26 (63%) patients for both treatments. The median CTX AUC value for treatment 1 was 257 mmol/L per min (range, 156-689). Median CTX and thiotepa AUC values for treatment 2 were 553 mmol/L per min (range, 264-974) and 11 mmol/L per min (range, 3-77), respectively. Median urine CTX excretion was 23% (range, 4%-36%) for treatment 1 and 32% (range, 10%-46%) for treatment 2. Median total CTX dose minus urine CTX amounts (which represents active and inactive metabolites of CTX in the blood) were 4454 mg (range, 3750-5090 mg) for treatment 1 and 5902 mg (range, 5338-6729 mg) for treatment 2. Median CTX AUC values were similar to their mean values (data not shown), whereas median thiotepa AUC was 11 versus a mean of 26. Thiotepa AUC values show a large interpatient variability, with a range of 3 to 77 mmol/L per min. The values shown in Figure 2 demonstrate an asymmetrical distribution skewed toward higher thiotepa AUC values.

Table 2. Toxicity (n = 47)*

Toxic death	1 (2)
Hospital days	
Total	32 (16-150)
After stem cell reinfusion	18 (6-139)
Hematologic toxicity	
Days to neutrophil count >500	15 (11-37)
Days to last platelet transfusion	11 (7-412)
Infectious complications†	
Episodes of neutropenic fever after treatment 1	13 (32)
Positive blood cultures after treatment 1	0 (0)
Positive blood cultures after treatment 2	5 (12)
Readmissions after BMT discharge‡	6 (15)
Gastrointestinal toxicity (n = 0)	
Days of hyperalimentation	16 (0-37)
Days of parenteral narcotic	11 (0-25)
Mucositis grade	
None	3 (8)
I	1 (3)
II	3 (8)
III	1 (3)
IV	32 (80)
Cardiac toxicity (n = 40)	
Left ventricular ejection fraction	
Pretreatment	59 (45-76)
Day 8	61 (36-72)
Postdischarge	60 (18-73)

*Data are n (%) or median (range).

†Treatment 1 consisted of cyclophosphamide 4 g/m² and treatment 2 consisted of cyclophosphamide 1.5 g/m² per day and thiotepa 200 mg/m² per day continuous infusion for 4 days.

‡Not related to disease recurrence.

Unpublished data from these and additional patients treated at this institution with the same dose and schedule of thiotepa and CTX without novobiocin demonstrated no alteration of thiotepa pharmacokinetics by novobiocin.

There was no statistically significant relationship between PFS and any of the AUC values, with *P* values of .71 and .74 for CTX (treatments 1 and 2, respectively) and .92 for thiotepa. Nor was any statistically significant relationship found with OS and CTX or thiotepa AUC values (*P* values .17 and .12 for CTX [treatments 1 and 2, respectively] and .42 for thiotepa). Median plasma 4HC/aldophosphamide AUC levels were comparable in both treatments at 6.3 and 6.9 mmol/L per min. There was no statistically significant relationship between 4HC/aldophosphamide AUC and PFS or OS. Risk ratios (confidence intervals) for 4HC/aldophosphamide for treatment 1 were 0.67 (0.14-2.75) and 0.70 (0.14-2.85) for OS and PFS, respectively. For treatment 2, they were 2.52 (0.61-12.51) and 3.81 (0.81-27) for OS and PFS, respectively.

In Vitro Reversal of Alkylator Resistance

Plasma samples from 12 patients were available for evaluation of in vitro reversal of alkylator resistance. Two of them had stage IIIB disease and 10 had stage IV. Two of 10 patients with stage IV disease had a CR to induction chemotherapy. Two of the 12 women remain disease free, 1 with stage IIIB disease and 1 with stage IV (who achieved a CR to induction chemotherapy).

Plasma samples collected after 36 hours of novobiocin therapy from all 12 patients were able to augment 4HC-induced cytotoxicity. The results from these studies are shown in Table 3 and Figure 3. Mean IC₅₀ values of 4HC treatment in the parental wild-type breast cancer cell line decreased 5.7-fold after exposure to post-novobiocin plasma samples. The CTX-resistant cell line also demonstrated an increased sensitivity to 4HC after exposure to the plasma collected after initiation of novobiocin. There was a 5.1-fold reduction in the mean IC₅₀ in this group, thus reducing the 4HC concentration required to achieve an IC₅₀ down to a comparable level in the pre-novobiocin group of the wild-type cell line (58 μmol/L and 60 μmol/L, respectively). Plasma derived from the 2 women who remain in CR did not demonstrate any consistently increased effect of novobiocin-augmented alkylator cytotoxicity in either cell line tested.

DISCUSSION

The role of high-dose therapy in the management of breast cancer remains to be defined. Conventional-dose chemotherapy produces response rates of 40% to 65% in women with metastatic disease, but only 10% to 25% are complete responses [34,35]. The duration of these responses is measured in months, and almost all patients die of their disease a median of 18 to 24 months from diagnosis [36,37]. High-dose therapy with PBSC or bone marrow support has resulted in increased response rates, including complete responses, but has not been shown to significantly alter median time to disease progression or overall survival compared with historical controls treated with conventional-dose chemotherapy [38]. Although 10% to 20% of the women treated with high-dose therapy appear to have durable complete responses, it is unclear if this is a larger fraction than might be observed in similar patients treated with standard-dose combination chemotherapy [39]. Indeed, a recent study by Stadtmauer et al. [40] randomized women with chemosensitive metastatic breast cancer to high-dose therapy and autologous transplant versus maintenance chemotherapy. The median OS and PFS of 24 months and 9.6 months, respectively, for the transplant group did not differ significantly from median OS and PFS of 26 and 9 months, respectively, for the maintenance group. Therefore, inherent or acquired drug resistance in this setting results in ultimate treatment failure for the majority of patients.

This study addressed whether the addition of novobiocin, an agent known to reverse alkylator drug resistance in experimental models, to a high-dose alkylator regimen in women with advanced breast cancer would suggest an increased clinical efficacy and warrant additional clinical evaluation. PFS or OS was not substantially altered by the addition of novobiocin to high-dose chemotherapy compared with historical controls treated with high-dose chemotherapy. An analysis by Antman et al. [6] of high-dose therapy for stage IV breast cancer using data derived from the Autologous Blood and Marrow Transplant Registry reported median OS and PFS of 18 and 9 months, respectively. In our study, the median OS was 21.5 months, and PFS was 10 months. Our study results therefore do not significantly differ from those obtained in patients treated with high-dose alkylator therapy alone in this historical

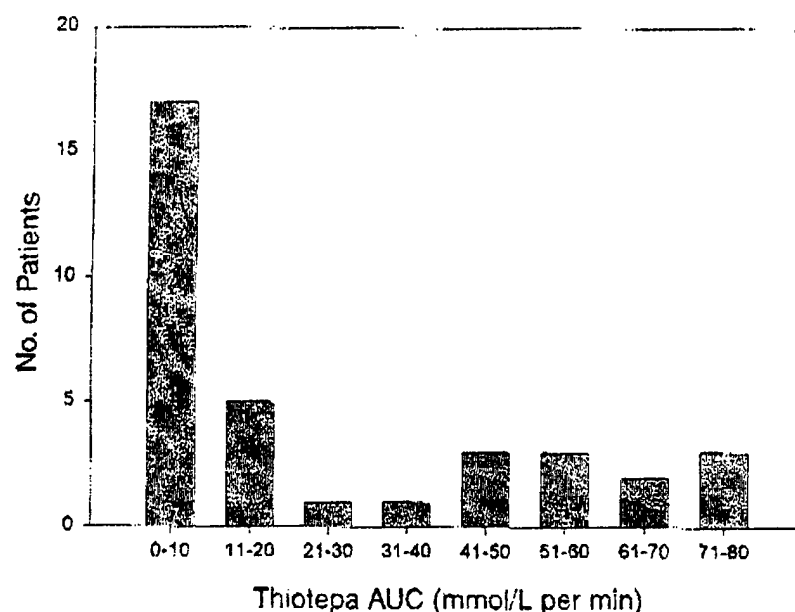


Figure 2. Distribution of thiotepa area-under-the-curve values ($n = 35$).

database [6,38]. These results also do not differ from OS and PFS for women with advanced breast cancer treated previously at our institution with an identical high-dose alkylator regimen (without novobiocin) and autologous marrow support (data not shown). Because eligibility criteria for trial entry and characteristics of this group of women were similar to those in our previous studies, selection bias is not likely to play a major role in this particular study. These results suggest a low likelihood that novobiocin's ability to reverse alkylator resistance had a significant benefit in terms of PFS or OS in this patient population. How-

ever, the small sample size cannot exclude the possibility of a small clinical benefit.

This study also evaluated the pharmacokinetics of CTX and thiotepa and analyzed their impact, if any, on PFS and OS. The metabolism of CTX is complex, and levels of the active product, phosphoramidate mustard, are determined by metabolism as well as urinary clearance of the inactive parent compound [41,42]. Studies have shown increased renal clearance of the parent compound in the setting of high-dose therapy because of the aggressive hydration used to prevent bladder toxicity [27,43].

Table 3. *In Vitro* Testing of Patient Plasma Samples for Reversal of Alkylator Resistance*

Patient	Stage	Response†	IC ₉₀ for 4HC (μmol/L)					
			Wild-Type MCF-7			CTX-Resistant MCF-7		
			Novobiocin		Fold Reduction	Novobiocin		Fold Reduction
			Before	After		Before	After	
1	IV	PR	71	11	6.5	340	54	6.3
2	IIIB†	—	60	14	4.3	290	70	4.1
3	IV	PR	52	12	4.3	334	41	8.1
4	IV‡	CR	49	4	12.3	290	68	4.3
5	IV	PR	52	9	5.8	252	68	3.7
6	IV	PR	55	3	18.3	245	60	4.4
7	IV	PR	55	9	6.1	290	66	4.4
8	IV	PR	64	7	9.1	305	57	5.4
9	IIIB	—	41	20	2.1	314	61	5.1
10	IV	PR	63	6	10.5	266	63	4.2
11	IV	CR	65	15	4.3	305	45	6.8
12	IV	PR	93	17	5.5	281	43	6.5
Mean ± SD			60.0 ± 13.2	10.6 ± 5.3	5.7	294.3 ± 26.9	58.0 ± 10.2	5.1

*4HC indicates 4-hydroperoxycyclophosphamide; CR, complete response; IC₉₀, drug concentration that inhibits 90% of colony formation in a clonogenic assay; IV, intravenous; PR, partial response.

†After induction chemotherapy.

‡Patients who remain in CR.

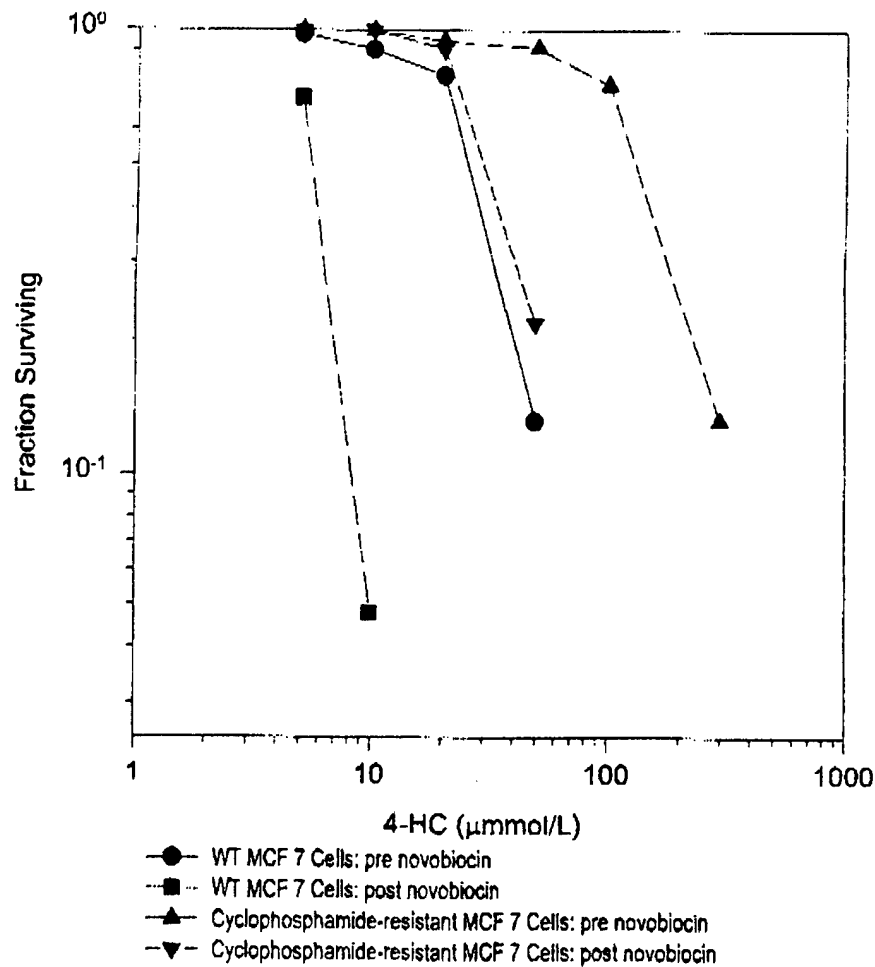


Figure 3. Actuarial progression-free and overall survival curves for evaluable patients (n = 41).

Figure 1 text

← move to under Figure 1

Therefore, significant amounts of the inactive parent compound can be cleared renally. In addition, there is significant interpatient variability in the metabolism of CTX [27,43,44]. Therefore, patient characteristics, renal clearance, concomitant medications, and metabolism all play a role in determining the extent of exposure to active drug for any patient.

CTX and thiotepa AUC values did not demonstrate a statistically significant correlation with PFS or OS in our study. Low CTX AUCs may be secondary to increased conversion into active metabolites, increased metabolism to inactive products, or renal clearance of the inactive parent compound. Therefore, the active metabolite 4HC was also measured in a subset of patients; again, no significant correlation with OS or PFS was observed.

Ayash et al. [45] showed that the median AUC values for CTX in patients treated with high-dose therapy for metastatic breast cancer were lower for the patients who developed clinical signs and symptoms of congestive heart failure (CHF). These same patients had not reached the median duration of response at a follow-up of 22 months, whereas patients who had not had CHF and had a higher median AUC had a median duration of response of only 5.25 months [45]. Ayash et al. hypothesized that increased

conversion of CTX to its active metabolites may have resulted in increased toxicity as well as efficacy, although metabolites or urinary levels of CTX were not determined. In contrast, our study failed to show any association between clinical benefit or toxicity and CTX or 4HC AUC values. The high-dose regimen in our study was not restricted to the carboplatin, CTX, and thiotepa regimen used by Ayash et al., and direct comparison of the 2 trials is thus difficult. Nonetheless, our results would argue against using CTX or 4HC values to predict patient response or toxicity.

Thiotepa levels demonstrated large interpatient variability and an asymmetric distribution. The skewed distribution of values in this population may be secondary to genetic differences in drug metabolism. Although this distribution was observed, its clinical relevance remains unclear because thiotepa AUC values did not correlate with a clinical response as measured by PFS or OS.

In addition, this study evaluated the ability of patient plasma samples obtained during novobiocin treatment to reverse CTX drug resistance in vitro. The assay measured CTX-induced cytotoxicity in both CTX-sensitive and -resistant breast cancer cell lines treated with patient plasma samples obtained before and during novobiocin therapy. Treatment of the cells in vitro with these samples resulted in a

2- to 18-fold decrease of IC_{50} in the sensitive cell line and a 4- to 8-fold decrease of CTX required to achieve an IC_{50} in the resistant cell line. Treatment of the CTX-resistant cell line resulted in almost 1 additional log cell kill. This level of activity is consistent with preclinical models.

However, these *in vitro* results did not translate into any apparent significant clinical advantage in terms of PFS and OS. There are several possible explanations for the lack of correlation between reversal of alkylator resistance *in vitro* and clinical response. First, these assays were accomplished in only 12 of 41 patients. These small numbers may not allow for a clinical advantage to be appreciated. In addition, only 2 of the 12 women are long-term disease-free survivors, making it difficult to comment on any relationship between clinical outcome and *in vitro* cytotoxicity. Also, the patient population included women with advanced breast cancer previously treated with chemotherapy, a time when tumor is likely made up of a mixed population of cells manifesting multiple genetic and biochemical abnormalities, which could result in drug resistance. Although these patients had chemosensitive disease, these molecular changes were likely associated with substantial chemotherapy resistance as suggested by the high relapse rate. Thus, it is possible that the presumed 1 to 2 log increase in CTX-induced cytotoxicity induced by novobiocin in this study was still insufficient to overcome drug resistance in a clinically relevant way in this group of women with heterogeneous advanced disease. Also, the effects of novobiocin were demonstrated indirectly in an *in vitro* assay using established cell lines rather than directly in an assay against patients' own tumor cells. The *in vitro* evaluation is a surrogate measure of patients' tumor response; only actual measurement of tumor cell resistance to alkylator therapy before and after novobiocin therapy using serial tumor samples from individual patients would provide direct evidence of novobiocin effects in tumor cells. Finally, although dose intensity is important in the treatment of breast cancer, there may be a dose plateau beyond which increased dose does not translate into clinical benefit [46,47]. In that case, modulating agents such as novobiocin might be more useful with standard-dose rather than high-dose therapy.

Although comparison of these results with historical controls is limited by many confounding factors, our results do not support evaluation of novobiocin in combination with high-dose alkylator therapy in metastatic breast cancer in a phase 3 clinical study. However, additional evaluation of the role of this agent or other compounds that reverse drug resistance may be warranted in the setting of women with advanced breast cancer who have minimal residual disease or in the adjuvant setting for high-risk patients. Indeed, in this study, the women who continue in CR had either stage IIIB disease (2 patients), stage IV NED, or stage IV disease in CR following induction chemotherapy or high-dose therapy ($n = 7$). The role of novobiocin, if any, in their response is impossible to assess because of the small number of women with these characteristics. Perhaps small increments in overall cell kill in this setting may translate to increased progression-free or overall survival. Additional phase 2 studies in this context may demonstrate increased clinical efficacy and warrant phase 3 evaluation.

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REFERENCES

1. Peters WP, Shpall EJ, Jones R, et al. High-dose combination alkylating agents with bone marrow support as initial treatment for metastatic breast cancer. *J Clin Oncol*. 1988;6:1368-1376.
2. Williams SF, Mick R, Desser R, Golick J, Beschmer J, Bitran JD. High-dose consolidation therapy with autologous stem cell rescue in stage IV breast cancer. *J Clin Oncol*. 1989;7:1824-1830.
3. Kennedy MJ, Beveridge RA, Rowley S, Gordon GB, Abeloff MD, Davidson NE. High-dose chemotherapy with reinfusion of purged autologous bone marrow following dose-intensive induction as initial therapy for metastatic breast cancer. *J Natl Cancer Inst*. 1991;83:920-926.
4. Antman K, Ayash L, Elias A, et al. A phase II study of high-dose cyclophosphamide, thiopeta, and carboplatin with autologous marrow support in women with measurable advanced breast cancer responding to standard-dose therapy. *J Clin Oncol*. 1992; 10:102-110.
5. Williams SF, Gilewski T, Mick R, Bitran JD. High-dose consolidation therapy with autologous stem-cell rescue in stage IV breast cancer: follow-up report. *J Clin Oncol*. 1992;10:1743-1747.
6. Antman KII, Rowlings PA, Vaughan WP, et al. High-dose chemotherapy with autologous hematopoietic stem-cell support for breast cancer in North America. *J Clin Oncol*. 1997;15:1870-1879.
7. Kennedy MJ, Armstrong DK, Huelskamp AM, et al. Phase I and pharmacologic study of the alkylating agent modulator novobiocin in combination with high-dose chemotherapy for the treatment of metastatic breast cancer. *J Clin Oncol*. 1995;13:1136-1143.
8. Kennedy MJ, Vogelsang GB, Jones RJ, et al. Phase I trial of interferon gamma to potentiate cyclosporine-induced graft-versus-host disease in women undergoing autologous bone marrow transplantation for breast cancer. *J Clin Oncol*. 1994; 12:249-257.
9. Dunphy FR, Spitzer G, Buzdar AU, et al. Treatment of estrogen-receptor negative or hormone refractory breast cancer with double high-dose chemotherapy intensification and bone marrow support. *J Clin Oncol*. 1990;8:1207-1216.
10. Hryniuk W, Bush H. The importance of dose intensity in chemotherapy of metastatic breast cancer. *J Clin Oncol*. 1984;2:1281-1288.
11. Hryniuk W, Levine MN. Analysis of dose intensity for adjuvant chemotherapy trials in stage II breast cancer. *J Clin Oncol*. 1986;4:1162-1170.
12. Teicher BA, Cucchi CA, Lee JB. Alkylating agents: *in vitro* studies of cross-resistance patterns in human cell lines. *Cancer Res*. 1986;46:4379-4383.
13. Frei E III, Canellos GP. Dose, a critical factor in cancer chemotherapy. *Am J Med*. 1980;69:585-594.
14. Marten MR, Scudiero DA. Characterization of the inhibition of replicative and repair type DNA synthesis by novobiocin and nalidixic acid. *Biochim Biophys Acta*. 1981;653:248-258.
15. Eder JP, Teicher BA, Holden SA, Cathcart KN, Schnipper LF. Novobiocin enhances alkylating agent cytotoxicity and DNA

- interstrand crosslinks in a murine model. *J Clin Invest*. 1987; 79:1524-1528.
16. Tan KB, Mattem MR, Boyce RA, Schein PS. Unique sensitivity of nitrogen mustard-resistant human Burkitt lymphoma cells to novobiocin. *Biochem Pharmacol*. 1988;37:4411-4413.
17. Lee FY, Flannery DJ, Siemann DW. Modulation of the cell cycle-dependent cytotoxicity of adriamycin and 4-hydroperoxycyclophosphamide by novobiocin, an inhibitor of mammalian topoisomerase II. *Cancer Res*. 1992;52:3515-3520.
18. DeJong S, Timmer-Bosscha H, DeVries F, Mulder NH. Effect of novobiocin on cisplatin cytotoxicity and DNA interstrand cross-link formation in a cisplatin-resistant, small-cell lung carcinoma cell line. *Int J Cancer*. 1993;53:110-117.
19. Rappa G, Lorico A, Sartorelli AC. Potentiation by novobiocin of the cytotoxic activity of etoposide (VP-16) and teniposide (VM-26). *Int J Cancer*. 1992;51:780-787.
20. Eder JP, Wheeler CA, Teicher BA, Schnipper LE. A phase I clinical trial of novobiocin, a modulator of alkylating agent cytotoxicity. *Cancer Res*. 1991;51:510-513.
21. Ellis GK, Crowley J, Livingston RB, Goodwin JW, Hutchins L, Allen A. Cisplatin and novobiocin in the treatment of non-small cell lung cancer. *Cancer*. 1991;67:2969-2973.
22. Oken MM, Creech RH, Tormey DC, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol*. 1982;5:649-655.
23. Yeager AM, Kaizer H, Santos GW, et al. Autologous bone marrow transplantation in patients with acute nonlymphocytic leukemia, using ex-vivo marrow treatment with 4-hydroperoxycyclophosphamide. *N Engl J Med*. 1986;315:141-147.
24. Passos-Coelho JL, Braine HG, Wright SK, et al. Large-volume leukapheresis using regional citrate anticoagulation to collect peripheral blood progenitor cells. *J Hematother*. 1995;4:11-19.
25. Passos-Coelho JL, Machado MA, Lucio P, Leal-Da-Costa F, Silva MR, Oarreira A. Large-volume leukaphereses may be more efficient than standard-volume leukaphereses for collection of peripheral blood progenitor cells. *J Hematother*. 1997;6:465-474.
26. Chen T-L, Passos-Coelho JL, Noe DA, et al. Nonlinear pharmacokinetics of cyclophosphamide in patients with metastatic breast cancer receiving high-dose chemotherapy followed by autologous bone marrow transplantation. *Cancer Res*. 1995; 55:810-816.
27. Chen T-L, Kennedy MJ, Anderson LW, et al. Nonlinear pharmacokinetics of cyclophosphamide and 4-hydroxycyclophosphamide/aldophosphamide in patients with metastatic breast cancer receiving high-dose chemotherapy followed by autologous bone marrow transplantation. *Drug Metab Dispos*. 1997; 25:544-551.
28. Cohen BE, Egorin MJ, Nayar MS, Gutierrez PL. Effects of pH and temperature on the stability and decomposition of N,N',N''-triethylenethiophosphoramidate in urine and buffer. *Cancer Res*. 1984;44:4312-4316.
29. Egorin MJ, Cohen BE, Kohlhepp FA, Gutierrez PL. Gas-liquid chromatographic analysis of N,N',N''-triethylene thiophosphoramidate and N,N',N''-triethylene phosphoramidate in biological samples. *J Chromatogr*. 1985;343:196-202.
30. O'Dwyer PJ, LaCreta F, Engstrom PF, et al. Phase I/pharmacokinetic reevaluation of thiotepa. *Cancer Res*. 1991;51:3171-3176.
31. Chiou WL. Critical evaluation of the potential error in pharmacokinetic studies using the linear trapezoidal rule method for the calculation of the area under the plasma level-time curve. *J Pharmacokinetic Biopharm*. 1978;6:539-546.
32. Anderson LW, Chen T-L, Colvin OM, et al. Cyclophosphamide and 4-hydroxycyclophosphamide/aldophosphamide kinetics in patients receiving high-dose cyclophosphamide chemotherapy. *Clin Cancer Res*. 1996;2:1481-1487.
33. Kaplan FL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc*. 1958;53:457-481.
34. Bull JM, Tormey DC, Li SH, et al. Randomized comparative trial of adriamycin vs methotrexate in combination drug therapy. *Cancer*. 1978;41:1649-1657.
35. Bonadonna G, Valagussa P. Chemotherapy of breast cancer: current views and results. *Int J Radiat Oncol Biol Phys*. 1983;9:279-297.
36. Mick R, Begg CB, Antman KH, Kornman AH, Frei E 3d. Diverse prognosis in metastatic breast cancer: who should be offered alternative initial therapies? *Breast Cancer Res Treat*. 1989;13:33-38.
37. Clark G, Sledge GW, Osborne CK, McGuire WL. Survival from first recurrence: relative importance of prognostic factors in 1,015 breast cancer patients. *J Clin Oncol*. 1987;5:55-61.
38. Smith GA, Henderson IC. High-dose chemotherapy (HDC) with autologous bone marrow transplantation (ABMT) for the treatment of breast cancer: the jury is still out. *Important Adv Oncol*. 1995;201-214. <<Proofreaders: this matches Medline.>
39. Rahman ZU, Frye DK, Buzdar AU, et al. Impact of selection process on response rate and long term survival of potential high-dose chemotherapy candidates treated with standard-dose doxorubicin containing chemotherapy in patients with metastatic breast cancer. *J Clin Oncol*. 1997;15:3171-3177.
40. Stadman EA, O'Neill A, Goldstein IJ, et al. Phase III randomized trial of high-dose chemotherapy (IDC) and stem cell support (SCT) shows no difference in overall survival or severe toxicity compared to maintenance chemotherapy with cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) for women with metastatic breast cancer who are responding to conventional induction chemotherapy: the 'Philadelphia' Intergroup Study (PBT-1). *Proc Am Soc Clin Oncol*. 1999;18:(abstr 1a).
41. Colvin M, Chabner BA. Alkylating Agents. In: Chabner BA and Collins JM, eds. *Cancer Chemotherapy*. Philadelphia: JB Lippincott; 1990.
42. Moore MJ. Clinical pharmacokinetics of cyclophosphamide. *Clin Pharmacokin*. 1991;20:194-208.
43. Busse E, Busch FW, Bohnenstengel F, et al. Dose escalation of cyclophosphamide in patients with breast cancer: consequences for pharmacokinetics and metabolism. *J Clin Oncol*. 1997;15:1885-1896.
44. Slattery JT, Kallhorn TF, McDonald GB, et al. Conditioning regimen-dependent disposition of cyclophosphamide and hydroxycyclophosphamide in human marrow transplantation patients. *J Clin Oncol*. 1996;14:1484-1494.
45. Ayash LJ, Wright JE, Tretyakov O, et al. Cyclophosphamide pharmacokinetics: correlation with cardiac toxicity and tumor response. *J Clin Oncol*. 1992;10:995-1000.
46. Davidson NE, Kennedy MJ. Dose-intensive chemotherapy for breast cancer: what is the evidence? In: Harris JR, Lippman ME, eds. *Diseases of the Breast Updates*, vol. 2, no. 3. NJ, Lippincott Williams & Wilkins, 1998.
47. Fisher B, Anderson S, DeCillis A, et al. Further evaluation of intensified and increased total dose of cyclophosphamide for the treatment of primary breast cancer: findings from National Surgical Adjuvant Breast and Bowel Project B-25. *J Clin Oncol*. 1999;17:3374-3388.

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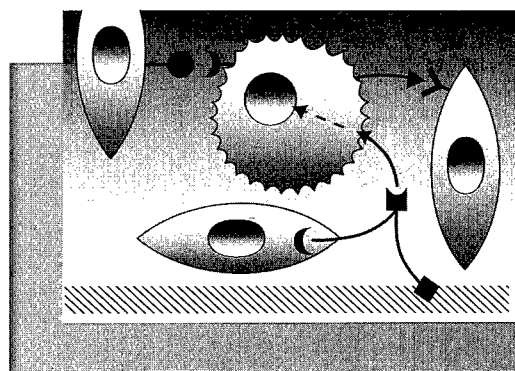
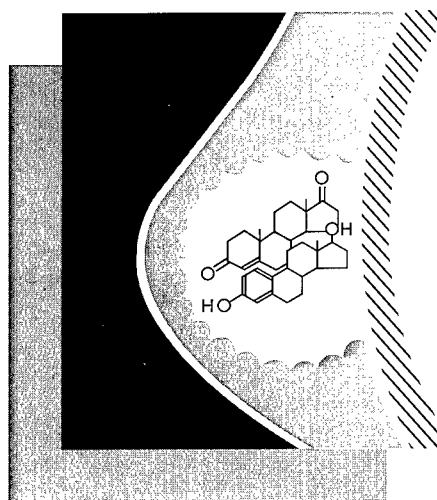
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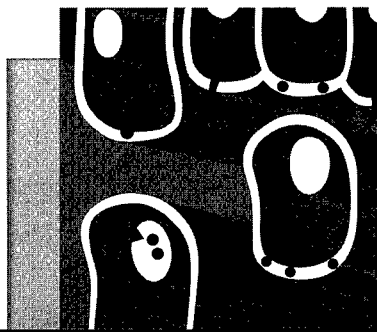
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Clinical aspects of cell death in breast cancer: the polyamine pathway as a new target for treatment

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Abstract

Because intracellular polyamines have a critical role in cell proliferation and death pathways, the polyamine metabolic pathway represents a potential target for intervention in cancers. A number of polyamine analogues have been identified that downregulate polyamine synthesis and enhance polyamine catabolism, thereby depleting intracellular polyamines. Treatment of human breast cancer cell lines in culture with these analogues has been shown to decrease cell proliferation and induce programmed cell death. Phase I studies with one analogue are now complete, setting the stage for phase II trials to determine efficacy, in addition to preclinical studies to examine combinations of polyamine analogues and conventional cytotoxics.

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Introduction

Metastatic breast cancer is a common disease. A major clinical problem is that tumours that are initially responsive to both hormonal and chemotherapeutic approaches generally progress to more aggressive forms that are poorly responsive to either category of agents. The need for anti-neoplastic agents with novel mechanisms of action is therefore great.

Intracellular polyamines have an important role in the proliferation of normal and malignant cells. The recognition of their critical role in cell growth and differentiation has led to the development of several inhibitors of polyamine biosynthesis, as exemplified by difluoromethylornithine (DFMO), which is directed against ornithine decarboxylase (ODC), the first enzyme in the polyamine biosynthetic pathway (Mamont *et al.* 1978, Danzin *et al.* 1982, Casero *et al.* 1984, Porter & Sufrin 1986, Marton & Pegg 1995). Both the chemotherapeutic and chemopreventive effects of DFMO have been the focus of several clinical trials.

Recently, however, attention has been focused on other steps in the polyamine metabolic pathway as potential targets for intervention. In particular, the *N,N'*-bis(ethyl)

analogues of spermine have been found to downregulate ODC, deplete intracellular polyamine pools, and inhibit cell growth (Porter *et al.* 1987). Depletion of polyamine pools and subsequent growth inhibition appear to be mediated in part through the induction of the enzyme, spermidine/spermine *N*¹-acetyltransferase (SSAT), the rate-limiting enzyme in the polyamine catabolic pathway (Porter *et al.* 1991, Casero & Pegg 1993). Although they readily accumulate in cells, a key feature of these analogues is that they do not substitute for the depleted natural polyamines (Porter *et al.* 1991). *N*¹-*N*¹²-bis(ethyl)spermine (BESpm) is a compound representative of this family of agents. In addition, several asymmetrically alkylated analogues that are structurally similar to the bis(ethyl)polyamines have been synthesised as potential anti-tumour agents (Saab *et al.* 1993). Structures for some of the substituted and unsubstituted polyamine analogues are shown in Fig. 1.

The importance of polyamine biosynthesis and action in breast cancer has been extensively studied and reviewed (Manni 1994). Because of these data, in addition to preclinical data suggesting anti-neoplastic effects of these symmetric and asymmetric polyamine analogues in a

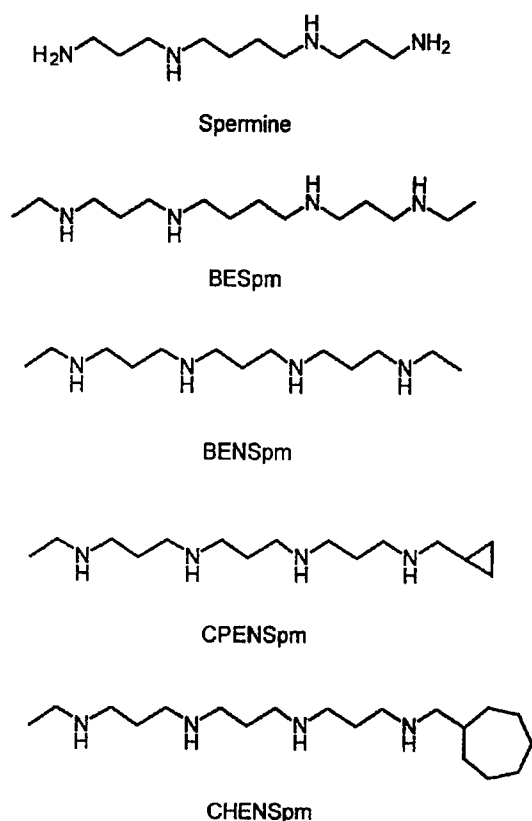


Figure 1 Structures of spermine and some polyamine analogues. BESpm, N¹,N¹²-bis(ethyl)spermine; BENSpm, N¹,N¹¹-bis(ethyl)norspermine; CPENSpm, N¹-ethyl-N¹¹-(cyclopropyl)methyl-4,8-diazaundecane; CHENSpm, N¹-ethyl-N¹¹-(cycloheptyl)methyl-4,8-diazaundecane.

variety of tumour types, including non-small cell lung cancer (Casero *et al.* 1989), melanoma (Bernacki *et al.* 1991), pancreatic cancer (Chang *et al.* 1991), and ovarian cancer (Bernacki *et al.* 1995), the effects of these compounds on the growth of human breast cancer cell lines in tissue culture have been assessed.

Growth effects of polyamine analogues

The prototype analogue, BESpm, significantly inhibits the growth of six human breast cancer cell lines (MCF-7, T47D, ZR-75-1, MDA-MB-231, MDA-MB-468, and Hs578t) with 50% inhibitory concentrations in the low micromolar range (Davidson *et al.* 1993). The degree of inhibition does not correlate with hormone receptor status. Detailed studies with the oestrogen receptor (ER)-positive MCF-7 and ER-negative Hs578t cell lines showed similar dose-response curves, with concentrations of 1-10 μ M resulting in maximal growth inhibition. Growth inhibition of both cell lines was associated with an 8- to 12-fold induction of the polyamine catabolic enzyme, SSAT, and progressive decrease in polyamine concentrations over 6 days, although steady-state concentrations of BESpm were achieved within 24 h. Similar studies on WTMCF-7 cells and the doxorubicin-resistant, ER-negative AdrMCF-7 cells derived from WTMCF-7 by step-wise incubation in doxorubicin show that acquisition of resistance to hormonal or doxorubicin treatment was not associated with resistance to the growth-inhibitory effects of BESpm. Indeed, the extent of growth inhibition, SSAT induction, and polyamine depletion after BESpm treatment was similar between the two cell lines. Thus, in aggregate, these initial results suggested that BESpm exerts similar growth-inhibitory effects against both

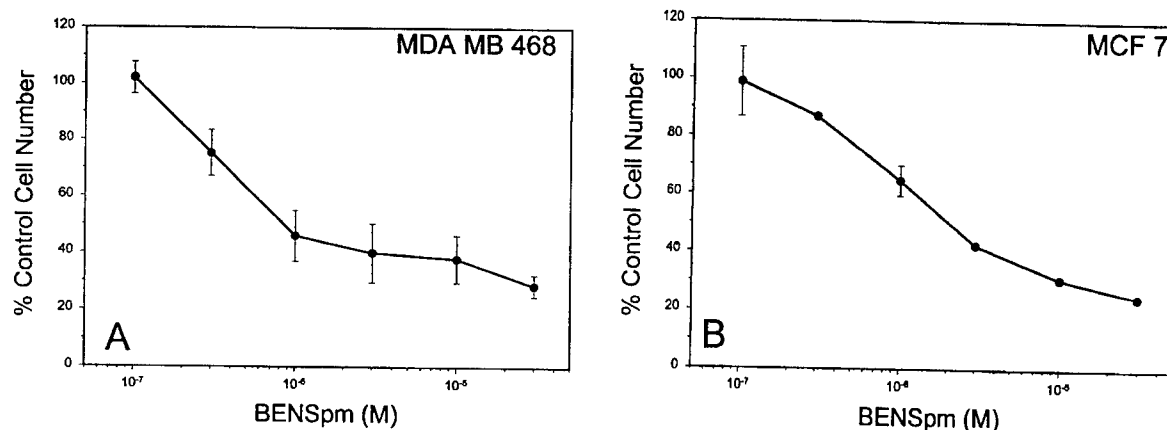


Figure 2 Dose-response curves for BESpm treatment of human breast cancer cells. MDA-MB-468 (A) and MCF-7 cells (B) in DMEM and 5% FCS were grown in the presence or absence of BESpm for 120 h. Cells were harvested and counted by Coulter counter; results are expressed as percentage of untreated control cell number. Means \pm S.D. for triplicate determinations from a representative experiment are shown.

Table 1 Effects of BENSpm on polyamine pools and SSAT activity in MDA-MB-468 cells

Treatment	Polyamines (nmol/mg protein)				SSAT activity (pmol/mg protein/min)
	Putrescine	Spermidine	Spermine	BENSpm	
Experiment 1					
Control	1.95	6.8	21	ND	82
BENSpm	0.96	0.86	4.6	40.0	34 778
Experiment 2					
Control	2.15	8.6	25	ND	55
BENSpm	1.6	1.4	5.7	30.0	21205

After incubation of exponentially growing MDA-MB-468 cells in the presence or absence of 10 μ M BENSpm for 24 h, intracellular polyamine concentrations and SSAT activity were determined as described previously (Davidson *et al.* 1993, McCloskey *et al.* 1995). Values are averages of two determinations from two independent experiments. ND, not determined.

hormone-responsive and -unresponsive human breast cancer cells. In addition, resistance to one chemotherapeutic agent, doxorubicin, was not associated with resistance of the polyamine analogue (Davidson *et al.* 1993).

The activity of the related compound, N^1 , N^{11} -bis(ethyl)norspermine or BENSpm (also known as N^1 , N^{11} -diethylnorspermine or DENSpm), has also been studied in selected human breast cancer cell lines. As shown in Fig. 2, treatment of MDA-MB-468 and MCF-7 cells resulted in growth inhibition, with an IC_{50} of 1-10 μ M after 120 h of chronic exposure. Treatment of MDA-MB-468 cells was associated with depletion of natural polyamines, intracellular accumulation of BENSpm, and a 400-fold induction of the polyamine catabolic enzyme, SSAT (Table 1). In addition, in preliminary studies, short-term explants of primary human breast cancers have been exposed to 10 μ M BENSpm overnight in tissue culture and evidence of induction of the SSAT enzyme has been observed by immuno-histochemistry (data not shown). Thus findings from established human breast cancer cell lines in culture appear also to hold true in short-term cultures of malignant breast tissue. The agent, BENSpm, is of particular interest, as it has been used in several phase I trials in humans, and a dose and schedule for phase II testing have been selected, as discussed below.

Polyamine analogues as mediators of programmed cell death

A relationship between polyamines and the process of programmed cell death has been suggested by several experimental findings. These include data suggesting that: (1) spermidine and spermine are able to stabilise chromatin (Marton & Morris 1987, Porter & Janne 1987),

(2) polyamine-depleted cells undergo changes in chromatin and DNA structure (Marton & Morris 1987, Porter & Janne 1987), and (3) spermine can protect against programmed cell death in thymocytes (Brüne *et al.* 1991). As a consequence, the possibility that polyamine analogues might induce programmed cell death in addition to their effects on proliferation was investigated, using an asymmetric polyamine analogue, N^1 -ethyl- N^{11} -((cyclopropyl)methyl)-4,8-diazaundecane (CPENSpm).

McCloskey *et al.* (1995) assessed the sensitivity to continuous CPENSpm exposure of the same six breast cancer cell lines evaluated in the BESpm study above. All cell lines exhibited concentration-dependent growth inhibition with IC_{50} values of 0.2-1.3 μ M. Again, there was no relationship between oestrogen receptor status and sensitivity to CPENSpm. Given the significant growth inhibitory activity of CPENSpm, the possibility that part of this effect was a result of induction of programmed cell death was investigated. Fragmentation of genomic DNA to high molecular weight fragments (≥ 50 kb) is characteristic of programmed cell death and may represent the committed step of the pathway. Field inversion gel electrophoresis was used to assess whether prolonged (4-day) exposure to CPENSpm could induce such DNA cleavage. Concentration-dependent high molecular weight DNA fragmentation was detected in all six cell lines after chronic exposure to CPENSpm and was not seen in untreated control cells. Because fragmentation of DNA into oligonucleosomal pieces is also associated with programmed cell death in many cell systems, DNA isolated from CPENSpm-treated MCF-7 and MDA-MB-468 cells was also examined for evidence of such a change. Oligonucleosomal DNA fragmentation was detected in both cell lines after exposure to 10 μ M CPENSpm for 96 h, but was not observed in untreated

control cells. Detailed studies using the MDA-MB-468 cell line confirmed that polyamine depletion and induction of SSAT were features of CPENSp_m-induced programmed cell death in this cell line. Interestingly, SSAT activity was not significantly induced in any of the other five breast cancer cell lines tested, suggesting that CPENSp_m cytotoxicity is not solely dependent on SSAT induction as a means of depleting polyamine.

Cytotoxic effects of CPENSp_m are not limited to breast cancer cell lines. Indeed, treatment of the NCI H157 human non-small-cell lung carcinoma cell line with either BESp_m or CPENSp_m resulted in polyamine depletion, SSAT induction, and morphological and biochemical changes consistent with activation of programmed cell death pathways (McCloskey *et al.* 1996). Studies using this cell line have also demonstrated that catalysis of polyamines by the SSAT/polyamine oxidase pathway has H₂O₂ as one product (Ha *et al.* 1997). Furthermore, the findings of studies utilizing inhibitors of this pathway suggested that programmed cell death induced by CPENSp_m may be due, in part, to oxidative stress as a result of H₂O₂ production (Ha *et al.* 1997). This observation is not characteristic of programmed cell death pathways induced by all polyamine analogues in this cell line, in that treatment with another asymmetrically substituted analogue, *N*¹-ethyl-*N*¹¹-(cycloheptyl-methyl)-4,8-diazaundecane (CHENSp_m), also led to high molecular weight DNA fragmentation, but through a mechanism that does not appear to involve oxidative stress; rather, CHENSp_m-treated, but not CPENSp_m-treated, H157 cells showed evidence of cell cycle changes - specifically, induction of a G₂/M block within 16 h of treatment (Ha *et al.* 1997). Thus it is likely that multiple mechanisms of action for these compounds exist.

Clinical application of polyamine analogues in breast cancer

Phase I testing of one polyamine analogue has now been completed. BENSp_m, also known as DENSp_m, has been studied in three different phase I studies, encompassing once-daily, twice-daily, and thrice-daily doses for 5 days of every 21-28-day cycle (Creaven *et al.* 1997, Ettinger *et al.* 1998, unpublished observations). Dose-limiting toxicities were predominantly those related to gastrointestinal or neurological toxicity. Haematological toxicity was not observed. These studies have led to identification of a dose of 100 mg/m² given as an intravenous bolus a single daily for 5 days of every 28 day cycle for use in phase II studies. Pharmacokinetic studies using this dose and schedule have shown that the maximal plasma concentration of BENSp_m achieved is 21-25 µM, a concentration that has been shown to have biological activity in preclinical models as summarised above. Thus phase II studies

examining the ability of BENSp_m to induce tumour regression or maintain a progression-free state can begin shortly.

Given the lack of haematological toxicity observed with BENSp_m in initial human studies, a second area for study is the possibility for combined therapy using polyamine analogues with more conventional cytotoxics already used for breast cancer treatment. Current studies are evaluating the effects in breast cancer cell lines in culture of combinations or sequences of polyamine analogues and agents such as doxorubicin, 5-fluorouracil, and the taxanes, looking for evidence of synergistic actions. It will then be possible to test promising combinations in animal model systems, in preparation for human trials.

Acknowledgements

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References

- Bernacki RJ, Bergeron RJ & Porter CW 1991 Antitumor activity of *N,N*¹-bis(ethyl) spermine homologues against human MALME-3 melanoma xenografts. *Cancer Research* **52** 2424-2430.
- Bernacki RJ, Oberman EJ, Seweryniak KE, Atwood A, Bergeron RJ & Porter CW 1995 Preclinical antitumor efficacy of the polyamine analogue *N*¹,*N*¹¹-diethylnorspermine administered by multiple injection or continuous infusion. *Clinical Cancer Research* **1** 847-857.
- Brüne B, Hartzell P, Nicotera P & Orrenius S 1991 Spermine prevents endonuclease activation and apoptosis in thymocytes. *Experimental Cell Research* **195** 323-329.
- Casero RA Jr & Pegg AE 1993 Spermidine-spermine *N*¹-acetyltransferase -the turning point in polyamine metabolism. *FASEB Journal* **7** 653-661.
- Casero RA, Bergeron RJ & Porter CW 1984 Treatment with α-difluoromethylornithine plus a spermidine analog leads to spermine depletion and growth inhibition in cultured L1210 leukemia cells. *Journal of Cellular Physiology* **121** 476-482.
- Casero RA, Ervin SJ, Celano P, Baylin SB & Bergeron RJ 1989 Differential response to treatment with bis(ethyl)polyamine analogues between human small cell lung carcinoma and undifferentiated large cell lung carcinoma in culture. *Cancer Research* **49** 639-643.
- Chang BK, Porter CW & Bergeron RJ 1991 Cellular responses to polyamine analogues and inhibitors in human pancreatic adenocarcinoma cell lines. *Journal of Cellular Pharmacology* **2** 133-137.
- Creaven PJ, Perez R, Pendyala L, Meropol NJ, Loewen G, Levine E, Berghorn E & Raghavan D 1997 Unusual central nervous system toxicity in a phase I study of *N*¹,*N*¹¹ diethyl-

- norspermine in patients with advanced malignancy. *Investigational New Drugs* 15 227-234.
- Danzin C, Bey P, Schirlin D & Claverie N 1982 α -Mono-fluoromethyl and difluoromethyl putrescine as ornithine decarboxylase inhibitors: *in vitro* and *in vivo* biochemical properties. *Biochemical Pharmacology* 31 3871-3878.
- Davidson NE, Mank AR, Prestigiacomo LJ, Bergeron RJ & Casero RA Jr 1993 Growth inhibition of hormone-responsive and -resistant human breast cancer cells in culture by N^1N^{12} -bis(ethyl)spermine. *Cancer Research* 53 2071-2075.
- Ettinger DS, Bowling K, Hoker B, Chen TL, Zabelina Y & Casero RA 1998 Phase I study of N^1N^{11} -diethylornspermine (DENSpm) in patients with non-small cell lung cancer (NSCLC). *Proceedings of the American Society of Clinical Oncology* 17 190A.
- Ha HC, Woster PM, Yager JD & Casero RA Jr 1997 The role of polyamine catabolism in polyamine analogue-induced programmed cell death. *Proceedings of the National Academy of Sciences of the USA* 94 11557-11562.
- McCloskey DE, Casero RA Jr, Woster P & Davidson NE 1995 Induction of programmed cell death in human breast cancer cells by an unsymmetrically alkylated polyamine analogue. *Cancer Research* 55 3233-3236.
- McCloskey DE, Yang J, Woster PM, Davidson NE & Casero RA Jr 1996 Polyamine analogue induction of programmed cell death in human lung tumor cells. *Clinical Cancer Research* 2 441-446.
- Mamont PS, Duchesne MC, Grove J & Bey P 1978 Anti-proliferative properties of DL- α -difluoromethylornithine in cultured cells. A consequence of irreversible inhibition of ornithine decarboxylase. *Biochemical and Biophysical Research Communications* 81 58-66.
- Manni A 1994 The role of polyamines in the hormonal control of breast cancer cell proliferation. *Cancer Treatment Reviews* 71 209-225.
- Marton LJ & Morris DR 1987 Molecular and cellular functions of the polyamines. In *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies*, pp 79-105. Eds PP McGann, AE Pegg & A Sjoerdsma. New York: Academic Press.
- Marton LJ & Pegg AE 1995 Polyamines as targets for therapeutic interventions. *Annual Review of Pharmacology and Toxicology* 35 55-91.
- Porter CW & Sufrin J 1986 Interference with polyamine biosynthesis and/or function by analogs of polyamines or methionine as a potential anticancer chemotherapeutic strategy. *Anticancer Research* 6 525-542.
- Porter CW & Janne J 1987 Modulation of antineoplastic drug action by inhibitors of polyamine biosynthesis. In *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies*, pp 203-248. Eds PP McGann, AE Pegg & A Sjoerdsma. New York: Academic Press.
- Porter CW, McManis J, Casero RA & Bergeron RJ 1987 The relative abilities of bis(ethyl) derivatives of putrescine, spermidine, and spermine to regulate polyamine biosynthesis and inhibit cell growth. *Cancer Research* 47 2821-2825.
- Porter CW, Ganis B, Libby PR & Bergeron RJ 1991 Correlations between polyamine analogue-induced increases in spermidine/spermine N^1 -acetyltransferase activity, polyamine pool depletion, and growth inhibition in human melanoma cell lines. *Cancer Research* 51 3715-3720.
- Saab MH, West EE, Bieszk NC, Preuss CV, Mank AR, Casero RA, Jr & Woster PM 1993 Synthesis and evaluation of unsymmetrically substituted polyamine analogues as modulators of human spermidine/spermine- N^1 -acetyltransferase (SSAT) and as potential antitumor agents. *Journal of Medicinal Chemistry* 36 2998-3004.

Breast cancer biology blossoms in the clinic

New clinical reports demonstrate that targeting the biological pathways of hormone and peptide growth factor receptors holds promise for the prevention and treatment of breast cancer.

BREAST CANCER is a leading and feared cause of cancer deaths among women. The antiestrogen, tamoxifen, has been used in its treatment for more than 20 years and now recent clinical trials point to a promising role for tamoxifen and related agents in breast cancer prevention¹⁻³. In addition, decades of basic research in breast tumorigenesis have provided the springboard to explore novel therapeutic approaches directed at other biological targets within tumor cells. New results from two clinical studies using a humanized monoclonal antibody directed against the HER-2 protein (which is over-expressed in 30% of breast tumors) indicate that targeting peptide growth factor pathways may also be effective in breast cancer treatment⁴⁻⁵.

Estrogen is required for growth and survival of breast epithelium and certain breast cancers (particularly the 60% that express estrogen receptor α , ER) may retain this property. Meta-analysis of 55 randomized trials of adjuvant tamoxifen therapy in 37,000 women has now confirmed that this antiestrogen substantially improves the 10-year survival of women with early stage ER-positive tumors regardless of age or menopausal status⁶. In contrast, the overall effect of tamoxifen on ER-negative tumors is small. In many of these studies, the incidence of contralateral breast cancer was also significantly reduced, prompting three large clinical trials to investigate the capacity of tamoxifen to prevent breast cancer in high risk women.

Early results from the American P-1 trial, in which 13,000 women were followed for an average of 3.6 years, indicate that tamoxifen reduces the incidence of breast cancer by more than 40% in all age groups¹. Two similar tamoxifen prevention trials in Europe are still ongoing. In preliminary

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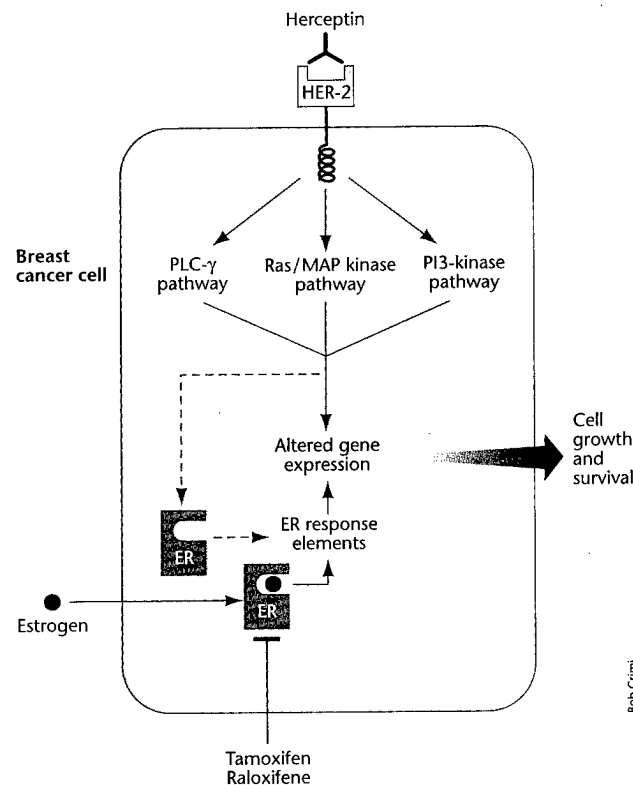
studies, raloxifene, another antiestrogen approved in the United States for treatment of osteoporosis, may also protect against breast cancer^{2,3}. Although the exact mechanisms by which these drugs might prevent breast cancer are not known, there are at least two possible explanations. A woman's risk of breast cancer is related to her lifetime exposure to estrogen, so effectively reducing that exposure may reduce breast tumorigenesis.

Alternatively, given the brevity of these trials and the finding that the incidence of ER-positive but not ER-negative tumors is reduced, the drugs may act as therapeutic agents on subclinical breast cancers. In either case, these studies provide the first tangible evidence that breast cancer may be partially preventable in the future.

Both tamoxifen and raloxifene exert their effects through their interaction with the ER (see Fig.), a transcription factor that interacts with numerous cofactors and binds to DNA response elements to modulate gene expression in a variety of tissues, including those of the breast,

bone and liver, and of the cardiovascular, urogenital and central nervous systems. Both tamoxifen and raloxifene are referred to as selective estrogen receptor modulators (SERMs) because they act as ER agonists in some tissues and ER antagonists in others. Ligand-dependent changes in conformation are thought to alter ER interactions with certain subsets of estrogen-responsive genes, thereby determining tissue-specific effects. For example, a single carbon atom in the hinge region of raloxifene appears to be responsible for its antagonistic effect on uterine tissues whereas tamoxifen, which lacks this carbonyl hinge, is uterotrophic⁷⁻⁸. However, both drugs act as ER antagonists in the breast and as agonists in skeletal and cardiovascular tissues. It is this high degree of selectivity that may allow these drugs to prevent breast cancer while promoting other beneficial effects such as maintenance of bone mineral density and diminished serum cholesterol levels.

Although the results of these studies are certainly cause for optimism, many questions remain about who to treat, which agent to give, when to administer therapy, and for how long. The rare adverse effects of tamoxifen (uterine can-



Possible molecular targets for breast cancer prevention or therapy. Approximately 60% of breast tumors are ER-positive and many of them are dependent on estrogen for growth and survival. Tamoxifen and raloxifene function as antiestrogens in breast cells by interacting with the ER without promoting transcription of essential estrogen-dependent genes. About 30% of breast cancers overexpress HER-2, a transmembrane receptor tyrosine kinase. Signal transduction following activation and tyrosine phosphorylation of HER-2 proceeds via several intracellular pathways as indicated. Overexpression and activation of HER-2 also leads to estrogen-independent transcriptional activity of ER and downregulation of ER protein levels (indicated by the dashed lines). Herceptin binds to the extracellular portion of HER-2, but the exact mechanism by which the antibody inhibits tumor growth is not known. (PLC- γ , phospholipase C- γ ; PI3-kinase, phosphatidylinositol 3 kinase; ER, estrogen receptor α).

Bob Crimi

cer and thromboembolic events) must be weighed against the potential benefits, and the long-term effects of raloxifene remain to be defined. Moreover, raloxifene has not been tested at all in premenopausal women. Additional antiestrogens that may offer better choices for breast cancer prevention in the future are under development. Ideally such an agent would act as an ER agonist in bone, liver, brain, heart and vagina, but as an antagonist in breast and endometrium.

Studies on the role of growth factor receptor pathways in breast cancer have led to development of therapeutic agents such as Herceptin, a monoclonal antibody that targets HER-2. About 30% of breast tumors overexpress HER-2, a transmembrane receptor tyrosine kinase. Signal transduction following activation and tyrosine phosphorylation of HER-2 proceeds via several intracellular pathways⁹ (see Fig.). Preliminary reports of Herceptin used alone⁴ or in combination with chemotherapy⁵ documented its efficacy against some breast cancers that express high levels of HER-2. Herceptin binds to the extracellular portion of HER-2, but the exact mechanism by which the antibody inhibits tumor growth is not known. Although some normal cells in the body also express HER-2, breast cancer cells may be more sensitive to these signaling pathways for growth and survival because, given alone, the antibody shows little toxicity in non-target tissues. However, Herceptin may potentiate the toxicity of chemotherapeutic agents. For example, concurrent administration of doxorubicin and Herceptin resulted in substantial cardiac toxicity⁵ as well as an improved tumor response rate.

Although preliminary, the results with Herceptin are of interest because they provide a new therapeutic option for some

patients for whom antiestrogen therapy is not effective. Many breast tumors that overexpress growth factor receptors are ER-negative and thus unresponsive to tamoxifen. Also acquisition of antiestrogen resistance in ER-positive tumors is common. Indeed, several small studies suggest that overexpression of HER-2 or a related tyrosine kinase receptor in ER-positive tumors may predict poor response to antiestrogens¹⁰. Those results are mirrored by the *in vitro* observation that cells forced to overexpress HER-2 become estrogen independent and tamoxifen resistant¹¹. Targeting other growth or survival pathways in these cancers with therapeutic agents such as Herceptin may provide a second line of attack.

The most exciting aspect of these initial studies is that they offer a paradigm for a potentially large new class of drugs. Several other growth factor receptors are overexpressed in subsets of breast tumors and thus may provide additional targets for antibody therapy. Vaccines, which would induce the patient's own immune system to generate a response against specific growth factor receptors, are also under investigation¹². Finally, rational drug design strategies may result in agents that inhibit specific growth factor receptors like HER-2 (ref. 13) or shared components of peptide receptor signaling pathways. Biologically based therapies in the form of antiestrogens have been a mainstay in breast cancer management. These new studies offer hope that therapeutic and prevention strategies directed at other molecular targets in breast cancer may also soon reach fruition.

1. Wickerham, D.L. *et al.* The initial results from NSABP protocol P-1: A clinical trial to determine the worth of tamoxifen for preventing breast cancer in women at increased risk. *Proc. Amer. Soc. Clin. Oncol.* **17**, 2a (Abstract #3a) (1998).
2. Cummings, S.R. *et al.* Raloxifene reduces the risk of breast cancer and may decrease the risk of en-

dometrial cancer in post-menopausal women. Two-year findings from the multiple outcomes of raloxifene evaluations (MORE) trial. *Proc. Amer. Soc. Clin. Oncol.* **17**, 2a (abstract # 3) (1998).

3. Jordan, V.C. *et al.* Incident primary breast cancers are reduced by raloxifene: integrated data from multicenter, double-blind, randomized trials in ~12,000 postmenopausal women. *Proc. Amer. Soc. Clin. Oncol.* **17**, 122a (Abstract #466) (1998).
4. Cobleigh, M.A. *et al.* Efficacy and safety of Herceptin (humanized anti-HER2 antibody) as a single agent in 222 women with HER2 overexpression who relapsed following chemotherapy for metastatic breast cancer. *Proc. Amer. Soc. Clin. Oncol.* **17**, 97a (Abstract #376) (1998).
5. Slamon, D. *et al.* Addition of Herceptin (humanized anti-HER2 antibody) to first line chemotherapy for HER2 overexpressing metastatic breast cancer (HER2+/MBC) markedly increases anticancer activity: A randomized, multinational controlled phase III trial. *Proc. Amer. Soc. Clin. Oncol.* **17**, 98a (Abstract #377) (1998).
6. Early Breast Cancer Trialists Collaborative Group. Tamoxifen for early breast cancer: An overview of the randomized trials. *The Lancet* **351**, 1451-1467 (1998).
7. Grese, T.A. *et al.* Molecular determinants of tissue selectivity in estrogen receptor modulators. *Proc. Natl. Acad. Sci. USA* **94**, 14105-14110 (1997).
8. Delmas, P.D. *et al.* Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. *N. Engl. J. Med.* **337**, 1641-1647 (1997).
9. Reese, D.M. & Slamon, D. HER-2/NEU signal transduction in human breast and ovarian cancer. *Stem Cells* **15**, 1-8 (1997).
10. Newby, J.C., Johnston, S.R.D., Smith, I.E. & Dowset, M. Expression of epidermal growth factor receptor and c-ErbB2 during the development of tamoxifen resistance in human breast cancer. *Clin. Cancer Res.* **3**, 1643-1651 (1997).
11. Pietras, R.J. *et al.* HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. *Oncogene* **10**, 2435-2446 (1995).
12. Disis M.L., Grabstein K.H., Sleath P.R. & Cheever M.A. HER-2/Neu peptide vaccines elicit T cell immunity to the HER-2/Neu protein in patients with breast and ovarian cancer. *Proc. Amer. Soc. Clin. Oncol.* **17**, 97a (Abstract #375) (1998).
13. Singh, J. *et al.* Structure-based design of a potent, selective, and irreversible inhibitor of the catalytic domain of the ErbB receptor subfamily of protein tyrosine kinases. *J. Med. Chem.* **40**, 1130-1135 (1997).

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International health beyond 2000

In the second Leverhulme lecture, David Nabarro (Department for International Development) discusses the lessons learnt from health issues of the last 20 years and the challenges facing health care in the 21st century.

THE 1998 WORLD Health Report 'Life in The 21st Century—A Vision for All', published by the World Health Organization in May this year, paints a picture of an "unmistakable trend towards healthier, longer life due to social and economic advances in the world in the late 20th century." The two main trends are increasing life ex-

DAVID NABARRO

pectancy and falling fertility rates, with the world's population now estimated to be eight billion by the year 2525. These trends reflect changing economic circumstances for people worldwide, greater availability of family planning

services, and some highly effective public health initiatives, including global immunization for all children. Family planning represents one of the extraordinary successes of international health, with the Children by Choice not Chance program making a major contribution to contraceptive prevalence and fertility reduction in Africa in recent years.

DIAGNOSIS IN ONCOLOGY

Arthur Skarin, MD, Consultant Editor

Breast Cancer Metastatic to the Choroid

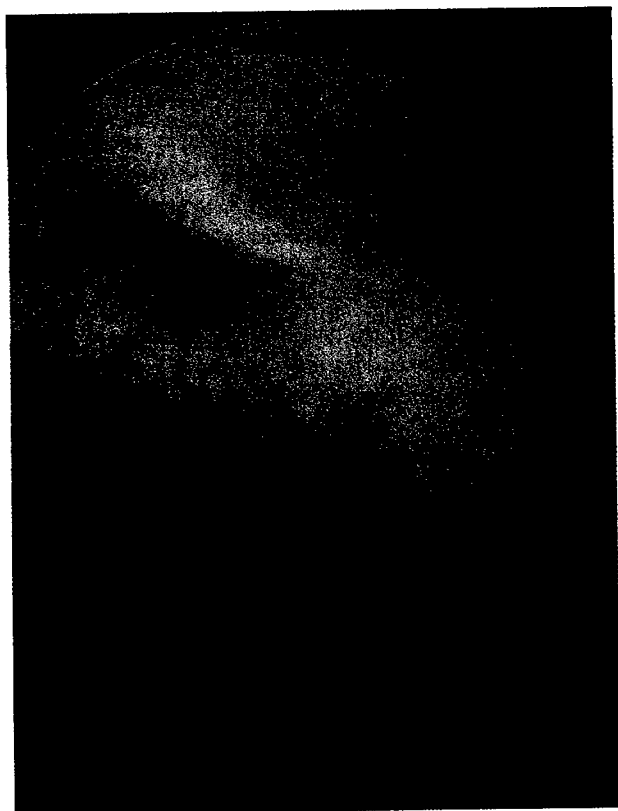


Fig 1.

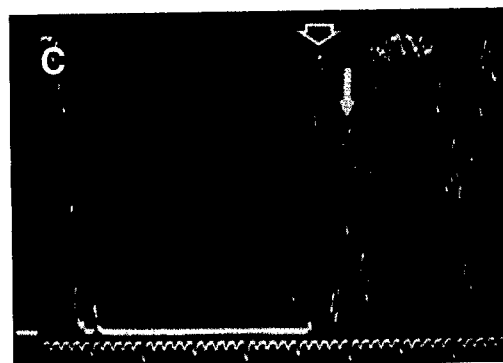
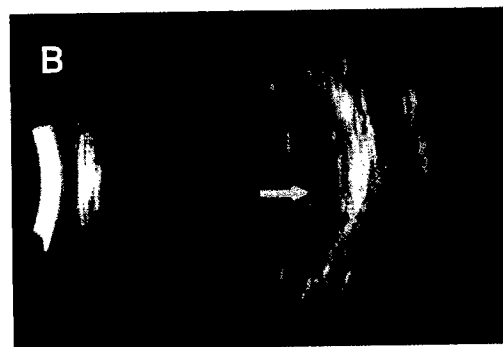
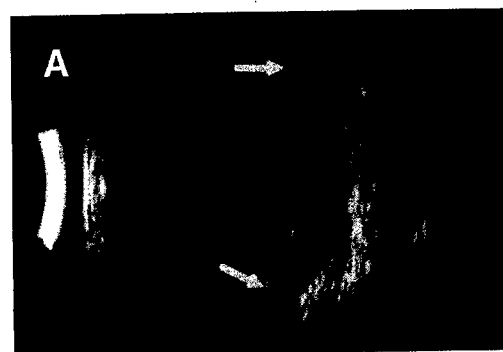


Fig 2.

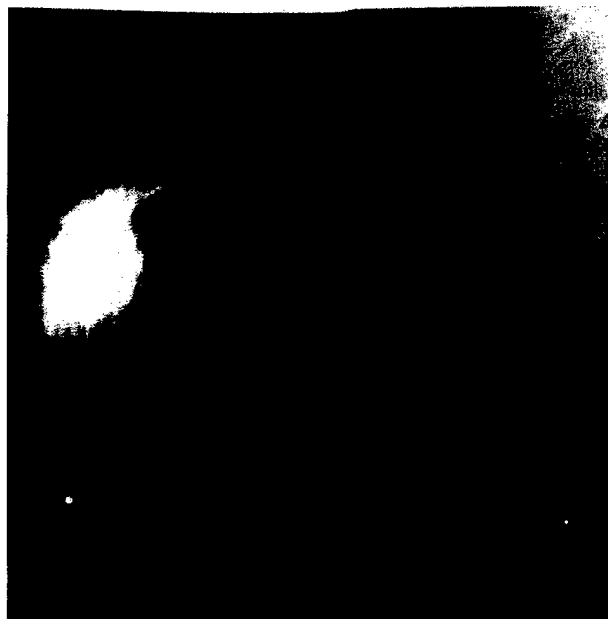


Fig 3.



Fig 4.

Several months after the death of her mother from breast cancer, a 51-year-old premenopausal woman presented to an ophthalmologist with complaints of blurred vision. A retinal detachment was observed on direct funduscopy (Fig 1). Ocular ultrasound examination is shown in Fig 2. Figure 2A is a tranverse (cross-section) B-scan that shows the lateral extent of a dome-shaped tumor that involves the inferotemporal fundus (arrows indicate the superior and inferior borders of the lesion). Figure 2B is a longitudinal (radial-section) B-scan. It is common for metastatic lesions to appear lobulated with an area of central excavation (arrow). An area of retinal detachment overlying the posterior aspect of the tumor also is seen. Figure 2C is a standardized A-scan that shows the surface of the tumor (open arrow) and the irregular internal structure (solid arrow). The reflectivity is determined by the histopathology of a given tumor. Metastatic lesions most frequently are comprised of cells with size heterogeneity, and interfaces that are not uniformly distributed throughout the tumor account for the irregularity of the internal spikes.

The patient was referred to an internist who palpated a solitary 5×3.5 cm left breast lump that was subsequently confirmed by mammogram (Fig 3). The patient had detected the lump several months previously, but had not reported it. Fine needle aspiration cytology showed adenocarcinoma. On thoracic computed tomographic evaluation, a small right pleural effusion and mediastinal metastatic disease were observed, as well as the primary breast tumor (Fig 4). Subsequently, the patient developed postobstructive pneumonia secondary to bulky mediastinal adenopathy. Systemic chemotherapy was initiated followed by hormonal therapy and chest wall and mediastinal irradiation. The patient's visual symptoms responded to chemotherapy and have remained stable over 2 years. Ocular radiation therapy was not used secondary to the urgent need for systemic chemotherapy and the subsequent improvement in the ocular symptoms. Estrogen and progesterone receptor status was never determined, but after systemic chemotherapy the patient was empirically placed on hormonal therapy and remains without signs of clinical progression 2 years later.

Choroidal metastases have been observed in approximately 8% of patients with metastatic breast cancer,¹ and in one third of these patients may be the first clinically detected site of relapse, as in this patient.² It has been recognized that breast cancer is the most common tumor type to metastasize to the choroid.³ Typically, these tumors manifest as either metamorphosis (distorted vision), reported as blurred vision, increased hyperopia or decreased myopia secondary to elevation of the macula, and decreased acuity in patients with previous visual problems.⁴ The true incidence of these tumors is likely to be higher than 8%, as they are not routinely evaluated in the absence of symptoms. Indeed an incidence of up to 37% has been reported in autopsy series.⁵ An association with cerebral metastasis has been reported in some,⁵ but not all series.² Cerebral metastases were not observed in our patient.

Radiotherapy is the treatment of choice for the palliation of symptomatic choroidal metastases with response rates of up to 80%.⁶

As in this patient, chemotherapy also has been successfully used.⁷ The prognosis for such patients traditionally has been considered to be poor, with reported average survival of 1 year.²

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REFERENCES

1. Weiss L: Analysis of the incidence of intraocular metastasis. *Br J Ophthalmol* 77:149-151, 1993
2. Doig RG, Olver IN, Jeal PN, et al: Symptomatic choroidal metastases in breast cancer. *Aust N Z J Med* 22:349-352, 1992
3. Orenstein MM, Anderson DP, Stein JJ: Choroid metastasis. *Cancer* 29:1101-1107, 1972
4. Mewin L, Young S: Breast carcinoma metastatic to the choroid. *Ophthalmology* 89:147-151, 1982
5. Bloch RS, Garner S: The incidence of ocular metastatic carcinoma. *Arch Ophthalmol* 85:673-675, 1971
6. Thatcher N, Thomas PRM: Choroidal metastases from breast carcinoma: A survey of 42 patients and the use of radiation therapy. *Clin Radiol* 26:549-553, 1975
7. Letson AD, Davidorf FH, Bruce RA: Chemotherapy for treatment of choroidal metastasis from breast carcinoma. *Am J Ophthalmol* 93:102-106, 1982